FACULTY OF MEDICINE AND HEALTH SCIENCES

Kinetics of protein-bound uremic toxins in chronic kidney disease

Olivier Deltombe

Promotor: Prof. dr. Sunny Eloot
Co-promotor: Prof. dr. Annemieke Dhondt

Thesis submitted to fulfill the requirements for the degree of 'Doctor in Medical Sciences'

Academic year: 2017 - 2018

GHENT UNIVERSITY

Members of the jury

- Prof dr. J. Vande Walle (chair)
- Prof. dr. S. Van Laecke (secretary)
- Prof. em. dr. R. Vanholder
- Prof. dr. A. Verstraete
- Dr. L. Balcaen
- Prof. dr. B. Bammens
- Prof. dr. P. Colin

Suggested citation: Deltombe, Olivier. 2018. "Kinetics of protein-bound uremic toxins in chronic kidney disease", PhD Dissertation. Ghent, Belgium: Ghent University. Faculty of Medicine and Health Sciences

Table of contents

List of abbreviations

A	
AAG	α_1-acid glycoprotein
ADMA	Asymmetric dimethylarginine
AIC	Akaike information criterion
APD	Automated peritoneal dialysis
ANOVA	Analysis of variance
B	
β_2M	β_2-microglobulin
BMI	Body mass index
BW	Bodyweight
C	
CAPD	Continuous ambulatory peritoneal dialysis
CMIA	Chemiluminescent microparticle immunoassay
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration
Crea	Creatinine
CV	Coefficient of variation
D	
Da	Dalton
DAD	Diode array detector
DC	Direct current
DIDS	4,4’-diisothiocyanato-2,2’-stilbenedisulfonic acid
DM	Diabetes mellitus
DTPA	Dietylenediaminepentaacetic acid
E	
ED	Equilibrium dialysis
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
EMA	European medicines agency
EPO	Erythropoietin
ESI (+ or -)	Electrospray ionization (in positive or negative mode)
ESKD	End-stage kidney disease

EUTox	European Uremic Toxin Workgroup
F	
FLD	Fluorescence detection
FPIA	Fluorescence polarization immunoassay
G	
GFR	Glomerular filtration rate
H	
HA	Hippuric acid
HCO	High cut-off
HD	Hemodialysis
HDF	Hemodiafiltration
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HESI (+ or -)	Heated electrospray ionization (in positive or negative mode)
HF	Hemofiltration
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HSA	Human serum albumin
I	
IAA	Indole-3-acetic acid
IS	Indoxyl sulfate
IL-6	Interleukin-6
Int. std.	Internal standard
K	
κ_{fl}	κ free light chains
KDIGO	Kidney disease: improving global outcomes
KDOQI	Kidney disease outcomes quality index
L	
λ_{flc}	λ free light chains
LOQ	Limit of quantification
M	
m/z	mass-to-charge ratio
MCO	Medium cut-off
MDRD	Modified diet in renal disease
MF	Matrix factor

MRM	Multiple reaction monitoring
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut-off
N	
NSA	Non-specific adsorption
O	
ODS	Octadecylsilane
P	
PAA	Phenyl acetic acid
PBUT	Protein-bound uremic toxin
PBS	Phosphate buffered saline
*p*CG	*p*-Cresyl glucuronide
*p*CS	*p*-Cresyl sulfate
PD	Peritoneal dialysis or pharmacodynamics
PDA	Photodiode array detector
PK	Pharmacokinetics
PTM	Post-translational modification
Q	
QC	Quality control
QCH	Quality control high
QCM	Quality control medium
QCL	Quality control low
QMS	Quantitative microsphere system
R	
RBC	Red blood cell
RF	Radiofrequency
RLU	Relative light unit
RP	Reversed phase
RT	Room temperature
S	
S_{crea}	Serum creatinine concentration
SDMA	Symmetric dimethylarginine

T

TDM	Therapeutic drug monitoring
Teico	Teicoplanin
TNFα	Tumor necrosis factor α

U

UA	Uric acid
UF	Ultrafiltration
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
UV – VIS	Ultraviolet – visible

V

Vanco	Vancomycin

Chapter 1

General introduction, aims and outline

1.1 Renal physiology and pathology

1.1.1 Normal kidney function

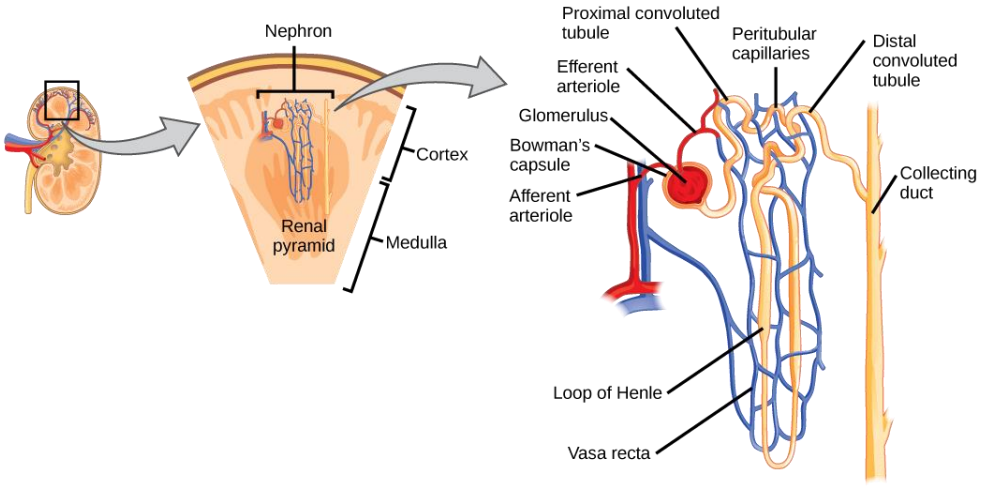
The kidneys are a pair of bean-shaped organs located in the retroperitoneal space of the abdominal cavity. Their main function is to excrete (i) metabolic end-products, like urea (protein metabolism) and uric acid (nucleic acid metabolism) and (ii) inorganic compounds from dietary intake (water, Na^+, PO_4^{2-}, Ca^{2+}, acids, bases, …). The kidneys also have an endocrine function producing 1,25-dihydroxyvitamin D (*i.e.* the active form of vitamin D), renin, an enzyme involved in blood pressure regulation and erythropoietin (EPO), a protein stimulating the bone marrow to generate erythrocytes.

The smallest functional unit of a kidney is the nephron, which is composed of a glomerulus, Bowman's capsule, proximal tubule, Henle's loop and distal tubule (Figure 1-1). Each kidney contains approximately $1 – 1.3\ 10^6$ nephrons and one collecting duct drains multiple nephrons. Blood purification is the result of two processes: glomerular filtration and tubular secretion. An afferent arteriole delivers blood to the glomerulus. Glomerular filtration forms the primary urine, by the process of ultrafiltration, which is collected in the Bowman's space. This primary urine has a similar composition than plasma, but mainly contains compounds with a molecular weight (MW) < 60 – 65 kDa. Subsequently, many of the filtered electrolytes (Na^+, K^+, Ca^{2+}, HCO^{3-}, PO_4^{2-}), water, glucose, and amino acids are reabsorbed into the blood. This reabsorption

takes place at the proximal tubule, Henle's loop and distal tubule via peritubular capillaries which are supplied by the efferent arteriole, containing blood that previously left the glomerulus. Amongst others, this unfiltered blood transports compounds which cannot be (totally) excreted by glomerular filtration, but only (also) by tubular secretion. This process takes place at the peritubular capillaries and is mostly the result of active transport. Approximately 1 % of the primary urine will reach the bladder via the ureters. Finally, the bladder is voided via the urethra during urination.

In healthy young individuals, the glomerular filtration rate (GFR) is about 125 mL/min/1.73 m² and declines gradually from the age of 35 – 40 years on (about 0.5 – 1 mL/min/1.73 m² per year) [1,2].

Figure 1-1 Schematic representation of the kidney's functional unit: the nephron [3].



1.1.2 Chronic kidney disease

1.1.2.1 Definition

In 2002, the Kidney Disease Outcomes Quality Index (KDOQI) guidelines from the United States National Kidney Foundation defined chronic kidney disease (CKD) as either kidney damage (*i.e.* pathologic abnormalities in blood or urine tests or in imaging studies) or a GFR < 60 mL/min/1.73 m² over a period of at least three months [4]. CKD was classified into 5 stages, based on the GFR only. The prevalence of CKD in the worldwide population is estimated to be 5 – 18 %, as based on the GFR < 60 mL/min/1.73 m² criterion [5–9]. Next to GFR, albuminuria (*i.e.* abnormal albumin loss in the urine) was found to be associated with the progression of kidney disease. For this reason, an update of CKD classification was published

in 2011 by the Kidney Disease: Improving Global Outcomes (KDIGO) in which albuminuria stages and a renal diagnosis category were added next to GFR stages (Figure 1-2) [10]. Patients with CKD stage 5 (GFR < 15 mL/min/1.73 m²) may need renal replacement therapy such as kidney transplantation or dialysis in order to survive when their GFR further declines. This stage is also referred as end-stage kidney disease (ESKD).

Figure 1-2 Prognosis of chronic kidney disease (CKD) by glomerular filtration rate (GFR) and albuminuria categories.

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012				Persistent albuminuria categories (mg/g) Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				< 30	30 – 300	> 300
GFR Categories (mL/min/1.73m²) Description and range	G1	Normal or high	≥ 90			
	G2	Mildly decreased	60 – 89			
	G3a	Mildly to moderately decreased	45 – 59			
	G3b	Moderately to severely decreased	30 – 44			
	G4	Severely decreased	15 – 29			
	G5	Kidney failure	< 15			

Green: low risk (if no other markers of kidney disease, no CKD); yellow: moderately increased risk; orange: high risk; red: very high risk. Adapted from [11].

1.1.2.2 GFR determination

The GFR can be measured by urinary or plasma clearance of exogenous (*i.e.* administered) filtration markers, which can be radio-isotopically labeled. Ideally, these markers are inert, not bound to proteins and only cleared by glomerular filtration. Examples of exogenous markers include inulin, ^{51}Cr-ethylenediaminetetraacetic acid (EDTA), ^{125}I-iothalamate and ^{99}Tc-dietylenediaminepentaacetic acid (DTPA) [12,13].

These methods are however costly and labor intensive. Therefore, GFR is in practice estimated (eGFR) by measuring endogenous filtration markers and using their concentrations in eGFR formulae. Creatinine is the most widely used endogenous filtration marker and has a relatively constant within-subject production. Renal excretion of creatinine occurs mainly via glomerular filtration without tubular reabsorption. However, a small fraction of creatinine is also cleared by tubular secretion, accounting for 5 – 10 % of the urinary content and is different among subjects. Consequently, creatinine clearance is an overestimation of GFR and becomes more

important with lower GFR. Nevertheless, due to the cheap and easily accessible analysis of serum creatinine in routine laboratories, creatinine-based formulae are the most commonly used in the clinical setting. The Cockcroft-Gault (Eq. 1-1) equation [14], which estimates the creatinine clearance, was the first formula that was used in practice. Nowadays, eGFR is usually estimated by the Modified Diet in Renal Disease (MDRD, Eq. 1-2) [15,16] or the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI, Eq. 1-3) [17] formulae.

$$\text{Creatinine clearance} = \frac{(140\text{-age})\ BW}{72\ S_{crea}}\ (0.85 \text{ if F}) \qquad \text{(Eq. 1-1)}$$

$$eGFR = 186\ S_{crea}^{-1.154} age^{-0.203}\ (0.742 \text{ if F})\ (1.21 \text{ if B}) \qquad \text{(Eq. 1-2)}$$

$$eGFR = 141\ \min\left(\frac{S_{crea}}{\kappa},1\right)^{\alpha} \max\left(\frac{S_{crea}}{\kappa},1\right)^{-1.209} (0.993)^{age}\ (1.018 \text{ if F})\ (1.159 \text{ if B}) \qquad \text{(Eq. 1-3)}$$

Where creatinine clearance and eGFR are expressed in mL/min and mL/min/1.73 m², respectively, age in years and bodyweight (BW) in kg. S_{crea} is serum creatinine concentration (in mg/dL), F is female, B is black, κ is 0.7 (female) or 0.9 (male) and α is -0.329 (female) or -0.411 (male).

It should however be stressed that the renal elimination of waste products and/or metabolites is not only controlled by glomerular filtration, but also by tubular secretion. Therefore, in some cases eGFR might only partially reflect renal elimination of specific metabolites.

1.2 Renal replacement therapy

At end-stage kidney disease (ESKD), *i.e.* a GFR < 15 mL/min/1.73 m², patients may need renal replacement therapy in order to purify the blood from (accumulated) waste products and metabolites.

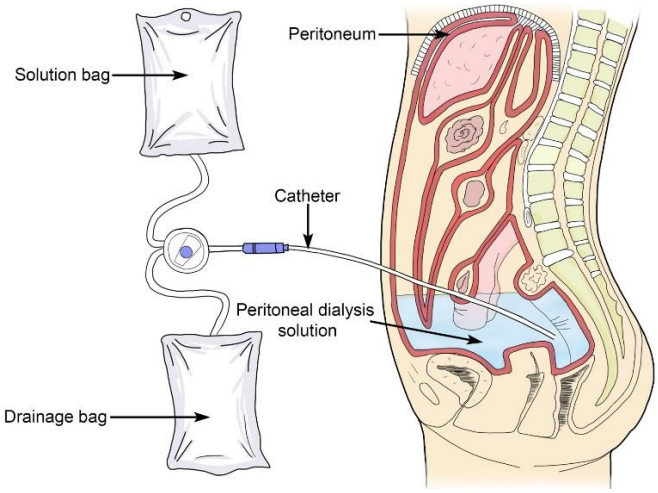
1.2.1 Kidney transplantation

Transplantation is, in general, the preferred renal replacement therapy. However, because of the lack of donor kidneys or transplant rejections/fails as well as the possibility that patients are not eligible for transplantation (e.g. due to comorbidity, infection or cancer), a significant portion of ESKD patients are treated with dialysis.

1.2.2 Peritoneal dialysis

In peritoneal dialysis (PD), the peritoneum is used as a semi-permeable membrane and allows the transport of small solutes, water and electrolytes (Figure 1-3). Via a permanent peritoneal catheter, placed in the lower part of the abdomen, the peritoneal cavity is filled with dialysis fluid. Small solutes move across the peritoneal membrane from blood side to dialysis fluid compartment, or vice versa, via diffusion, depending on the concentration gradient. Water removal takes place by osmosis as well, due to the addition of an osmotic agent (e.g. glucose, icodextrin, amino acids) to the dialysis fluid. In continuous ambulatory peritoneal dialysis (CAPD), the fluid is drained at regular intervals (typically 4 – 6 hours) and replaced by means of gravity, with a long dwell time overnight. In automated peritoneal dialysis (APD), several fluid changes are performed overnight, with a long dwell time during the day.

Figure 1-3 Schematic representation of peritoneal dialysis [18].



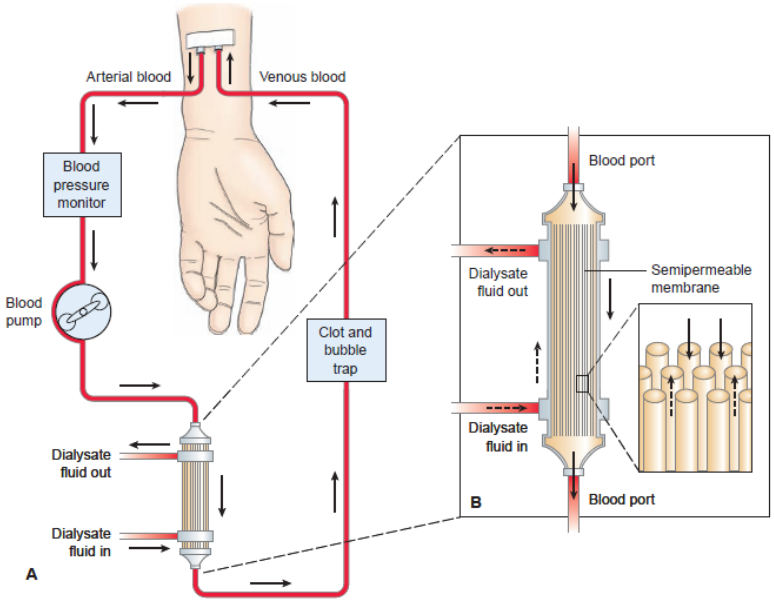
PD might be the first choice of treatment for several reasons. The continuous character of this treatment is less stressful for the heart and results in an improved well-being of the patient compared to hemodialysis (HD), where the treatment is intermittent and waste products accumulate between each dialysis session. Furthermore, PD is characterized by a lower cost for the society and PD patients have increased flexibility compared to those on hemodialysis.

1.2.3 Hemodialysis

1.2.3.1 Principle

In hemodialysis, blood flows through an artificial kidney (*i.e.* the hemodialyzer) in an extracorporeal circuit (Figure 1-4). A typical dialyzer contains about 10000 hollow fibers, having an internal diameter in the range of 180 – 225 µm and a wall thickness of 15 – 50 µm, depending on the brand [19–21]. Blood flows in the lumen of these hollow fibers, while dialysis fluid (*i.e.* pure water containing electrolytes) flows around them, in the opposite direction. These fibers act as a semi-permeable membrane and allow the transport of small compounds and water through the many pores via different transport mechanisms (see *1.2.3.3 Removal processes in hemodialysis*). Typical blood flow rates are in the range of 250 – 350 mL/min, whereas dialysis fluid flow rate is preferably 1.5 – 2 times the blood flow. As vascular access, an arteriovenous fistula or graft can be used as well as a central venous catheter. Next to the removal of waste products and metabolites, hemodialysis can also correct for electrolyte imbalance and acidosis by choosing the optimal dialysis fluid composition.

Figure 1-4 Schematic representation of hemodialysis [22].



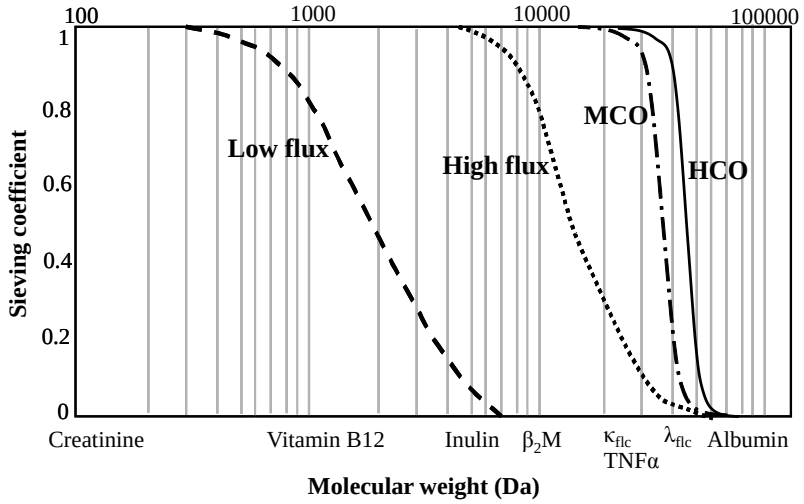
Typically, patients come to the hospital three times a week for a dialysis session of four hours. Not only night dialysis in the hospital can be an alternative, but also self-care or home dialysis treatment might be an option, especially for patients that go to work during the day. In between the different dialysis sessions, the plasma concentration of solutes increases due to

re-equilibration in the body immediately after dialysis (*i.e.* the so-called rebound) and due to generation after liquid and food intake, both depending on the residual renal function.

1.2.3.2 Dialyzer membranes

Low-flux hemodialysis treatment uses membranes with small pores (*i.e.* low flux), enabling removal of mainly small compounds, such as urea (60 Da) or creatinine (113 Da). The more efficient removal of larger solutes, such as β_2-microglobulin (β_2M, 12 kDa), can be established by using membranes with larger pores (*i.e.* high flux). The sieving coefficient describes the permeability of a hemodialyzer membrane for a solute and varies between 0 (not permeable) and 1 (fully permeable). In Figure 1-5, the sieving coefficient for a low and high flux membrane is presented as a function of solute molecular weight. More recently, membranes having larger pore sizes with a more uniform distribution (medium and high cut-off) became available. This results in an even higher permeability for high molecular weight solutes and steeper curves in Figure 1-5, respectively [23].

Figure 1-5 Sieving coefficient as a function of molecular weight for different hemodialysis membrane types, based on the pore size.



MCO: medium cut-off; HCO: high cut-off; β_2M: β_2-microglobulin, κ_{flc}: κ free light chains; TNFα: tumor necrosis factor α; λ_{flc}: λ free light chains (see *1.3.2 Middle molecules*). Adapted from [23].

Next to the variation in membrane thickness, and pore size, dialyzer membranes also differ in chemical composition. Most common synthetic dialyzer membranes are nowadays made of polysulfone, polyamide, polyethersulfone, polyacrylonitrile or a copolymer of polyarylethersulfone, polyvinylpyrrolidone and polyamide, depending on the brand [19–21].

To avoid blood clotting in the dialyzer, anticoagulants like low molecular weight heparin are usually administered at the start of the dialysis session.

1.2.3.3 Removal processes in hemodialysis

In the dialyzer, blood purification is the result of four physical processes, *i.e.* diffusion, convection, osmosis and adsorption. Diffusion is the result of solute concentration gradient between blood and dialysate across the semi-permeable membrane. Applying a countercurrent blood – dialysate flow, increases the removal of solutes due to an established concentration gradient over the entire length of the dialyzer. Convective transport of solutes and fluid removal are achieved by ultrafiltration induced by a transmembrane pressure gradient. This convective transport is, however, counteracted by osmosis due to the presence of proteins in blood. In the clinically used adsorptive hemodialysis techniques, solutes are removed from the blood by the adsorption to a resin in a separate column; i.e. hemodialysis with HFR Evolution (Bellco-Medtronic, Italy) [24] and liver dialysis with fractionated plasma separation and adsorption (FPSA) as with the Prometheus (Fresenius Medical Care, Germany) [25,26]. In addition, *in vitro* data showed that when adding sorbent to the dialysate side [27], using a monolithic sorbent [28] or combining hemodialysis with adsorption on one membrane (*i.e.* the mixed matrix membrane) [29] might be promising alternatives.

1.2.3.4 Different dialysis modes

When using large membrane pores, one is able to choose between different dialysis modes. In hemodialysis (HD), the main transport is diffusion and results in the clearance of mainly small solutes. A small portion of convective transport, either only forward filtration or both forward and backfiltration (*i.e.* internal filtration), takes place when correcting for the patient's dry bodyweight [30]. In hemofiltration (HF), there is only convection, removing substantial quantities of body fluid (*i.e.* plasma water), while substituted by buffered saline in pre- or post-dilution, *i.e.* at the arterial or venous blood line, respectively. Hemodiafiltration (HDF) combines both removal modes, *i.e.* diffusion and convection, resulting in a good removal of both small and larger compounds [31].

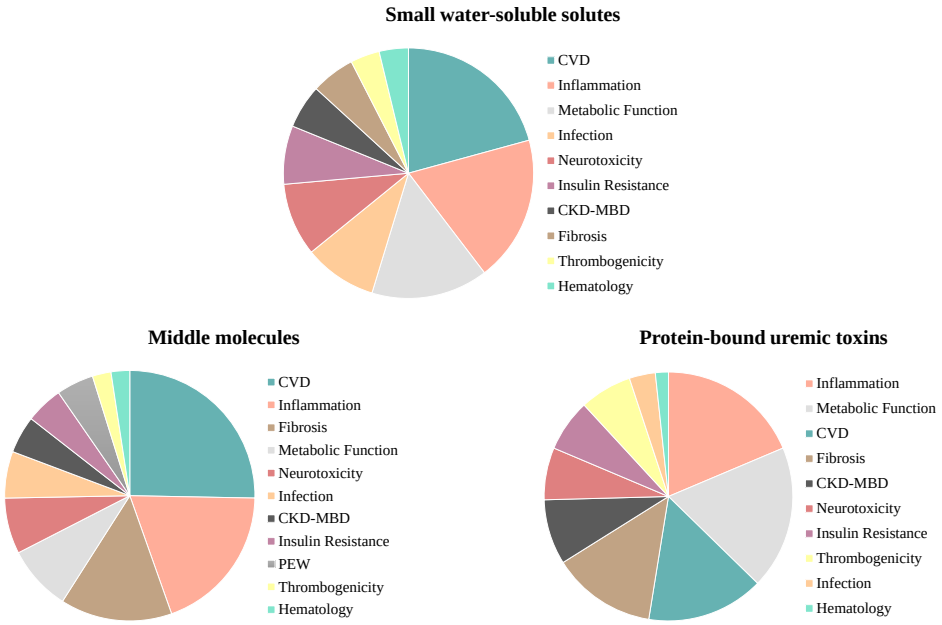
1.3 Uremic toxins

CKD is characterized by the retention of a large number of compounds that are normally excreted by the healthy kidneys. Many of these uremic retention solutes have a negative impact on biological functions of the body and are therefore called uremic toxins. Three groups of uremic toxins were proposed by the European Uremic Toxin (EUTox) Workgroup as based on their physicochemical properties, *i.e.* the small water-soluble solutes, middle molecules, and protein-bound uremic toxins [32,33].

1.3.1 Small water-soluble solutes

Small water-soluble solutes have a MW < 500 Da and are not bound to proteins. The prototype within this group is urea (60 Da). Uric acid (UA, 168 Da) and guanidine compounds such as creatinine (Crea, 113 Da), asymmetric dimethylarginine (ADMA, 202 Da) and symmetric dimethylarginine (SDMA, 202 Da) are also commonly studied [32–34]. Several small water-soluble solutes have been associated to cardiovascular disease, inflammation and metabolic function, as illustrated in Figure 1-6 [35].

Figure 1-6 Relative amount of toxins within each group of uremic toxins affecting specific biological systems.



CVD: cardio-vascular disease; CKD-MBD: chronic kidney disease-metabolic bone disease; PEW: protein energy wasting. Adapted from [35].

1.3.2 Middle molecules

Middle molecules are retention solutes in the large molecular weight range, *i.e.* > 500 Da, with β_2-microglobulin (β_2M, 11 kDa) as prototype. Amongst others, interleukin-6 (IL-6, 24.5 kDa), tumor necrosis factor α (TNFα, 26 kDa), immunoglobulin κ and λ free light chains (κ_{flc} and λ_{flc}, 22.5 kDa and 45 kDa respectively) are included in this class [32–34]. Many of these middle molecules have been linked to cardiovascular disease, inflammation and fibrosis (Figure 1-6) [35].

1.3.3 Protein-bound uremic toxins

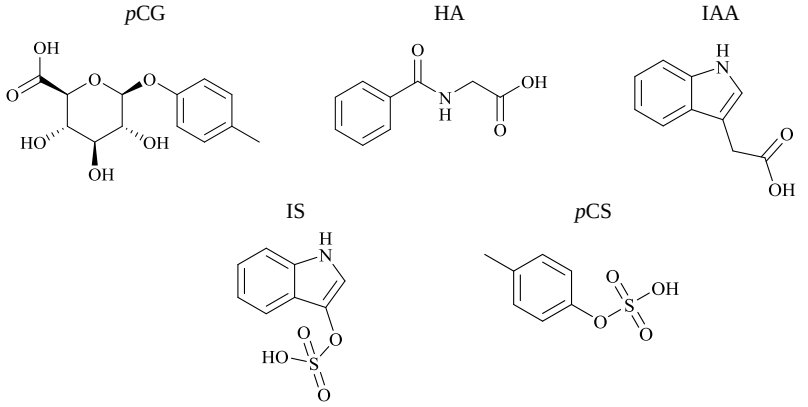
The protein-bound uremic toxins (PBUTs) are a group of compounds with predominantly a MW < 500 Da, where a certain fraction of their concentration is bound to plasma proteins. Prototypes of this group are phenols (e.g. *p*-cresyl sulfate) and indoles (e.g. indoxyl sulfate) [32–34]. In analogy with compounds from the other two classes, also a large number of PBUTs are associated to inflammation, metabolic function, cardiovascular disease and fibrosis, as indicated in Figure 1-6.

1.4 Kinetics of protein-bound uremic toxins

In this thesis, only a selected panel of PBUTs was studied, including *p*-cresyl glucuronide (*p*CG, 284 Da, pKa = 3.30), hippuric acid (HA, 179 Da, pK_a = 3.59), indole-3-acetic acid (IAA, 175 Da, pK_a = 4.66), indoxyl sulfate (IS, 213 Da, pK_a = -1.80) and *p*-cresyl sulfate (*p*CS, 188 Da, pK_a = -2.00) and their chemical structures are presented in Figure 1-7. All five listed PBUTs are organic acids and ionized for > 99.9% at physiologic pH (*i.e.* pH = 7.4), as calculated from their pK_a – values (Eq. 1-4).

$$\% \text{ ionized} = \frac{10^{pH-\,pK_a}}{10^{pH-\,pK_a+1}} \qquad \text{(Eq. 1-4)}$$

Figure 1-7 Chemical structures of *p*-cresyl glucuronide (*p*CG), hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS).



1.4.1 Generation and secretion

Phenolic and indolic compounds are predominantly generated by gut microbiota which takes place in the distal part of the colon [36,37]. Proteins, mostly originating from diet, enter the large intestine and are depolymerized into small oligopeptides and amino acids. The amino acids tyrosine and phenylalanine are further converted into phenolic compounds such as phenol and 4-methyl phenol, which is also known as *p*-cresol. Subsequently, *p*-cresol is conjugated by sulfotransferase and glucuronlytransferase in the mucosa of the colon and in the liver into *p*CS and, to a lesser extent, to *p*CG [38]. Indolic compounds, such as IAA and IS, are generated from the amino acid tryptophan by bacteria in the colon generating indole which is further metabolized in the liver into IS [39,40]. While the role of gut microbiota in the generation of *p*CG, IAA, IS and *p*CS in humans is well-documented, this is not the case for HA. It has been shown that benzoic acid, the precursor of HA, originating from plant polyphenols or from added food preservatives, is metabolized into HA. In animal studies, gut microbiota were found to be involved in this process [41,42] and human data presented by Aronov *et al*. suggested that the place of action in humans is in the small intestine rather than in the colon [30]. Besides benzoic acid, quinic acid, a sugar compound in plants, is believed to be metabolized into HA as well, after dietary intake [43].

All above described compounds bind to plasma proteins when entering the circulating system [44]. In normal conditions, the free (*i.e.* unbound) fraction of these PBUTs is cleared by glomerular filtration, whereas organic anion and cation transporters expressed on proximal epithelial cells of the tubule are responsible for the secretion of the protein-bound fraction [45].

1.4.2 Protein binding

The binding of PBUTs to plasma proteins is not covalently, but a result of intermolecular interactions between both. These interactions may include hydrophobic Van der Waals interactions, hydrogen bonds and/or electrostatic interactions with ionized residues, present on the toxin and plasma protein. Consequently, this binding is reversible and there is an equilibrium between bound and free fraction in plasma. The percentage that is bound to plasma proteins is expressed as the percentage protein binding (%PB) and ranges from almost 0 % to even 100 % [44]. Previous studies demonstrated that albumin, the most abundant protein in plasma (> 50 % of total protein), is the major binding protein for these PBUTs [46]. Next to albumin, these PBUTs can, in theory, also be bound to other plasma proteins, such as α_1-acid glycoprotein (AAG). However, to the best of our knowledge, no proof for binding to AAG or other plasma proteins has been published for PBUTs so far.

The free drug hypothesis states that for most drugs the free (*i.e.* unbound) concentration at the site of action exerts pharmacological activity [47]. Accordingly, it could be hypothesized that the toxicity of PBUTs is exerted by the free fraction rather than the total amount. In literature, it is often postulated that the %PB of uremic toxins might be decreased in patients with CKD as compared to healthy subjects, because of the possible saturation of plasma proteins as a consequence of the elevated uremic toxin concentrations in patients with CKD [48]. Additionally, it has been suggested that the %PB of PBUTs might be influenced by competition between different PBUTs bound to the same binding site on albumin [49–51], as well as between PBUTs and drugs like antibiotics [49,52–60]. If the %PB of PBUTs in patients with CKD would indeed be decreased due to protein saturation or binding competition, this may also have biological implications as a decrease from e.g. %PB = 95 % to 90 % means a doubling in free fraction and potentially a doubling in toxic effect. In clinical pharmacology, it has however been matter of debate whether alterations in %PB of drugs are clinically relevant and it is now believed that this is only the case for drugs with a narrow therapeutic window that are administered intravenously, having a high clearance and high %PB (*i.e.* > 70 %) [61].

1.4.3 Kinetics during dialysis

For many years, urea clearance (K) multiplied by time (t) and normalized for urea distribution volume (V_d) was the parameter to evaluate dialysis adequacy: $[Kt/V_d]_{urea}$. The choice for urea as a marker was, however, based on practical rather than on pathophysiologic considerations.

It should therefore be stressed that $[Kt/V_d]_{urea}$ only estimates the kinetics of urea, which is a small water-soluble compound that is easily removed by diffusion during dialysis and has a distribution volume close to the patient's total body water. Previous studies reported that this parameter does not adequately describe the removal of some other small water-soluble compounds [62–66] and certainly not of larger middle molecules and PBUTs, which are removed by different transport mechanisms and may have a different distribution volume as compared to urea [67–71].

It is therefore of great interest to study the kinetics of middle molecules and PBUTs during dialysis and finally find (a) representative compound(s) for each group of uremic toxins. For PBUTs, finding a representative compound might be more difficult than first thought, because the range of protein binding is large and only the free fraction can pass the pores of the dialyzer membrane.

1.5 Aims and outline

Because of the protein binding, removal efficiency of protein-bound uremic toxins (PBUTs) during hemodialysis (HD) is much lower as compared to small water-soluble solutes, especially for those with a high %PB. Since many of the PBUTs have an impact on the cardiovascular system and contribute to the increased propensity for cardiovascular events and mortality in patients with chronic kidney disease (CKD), it is of great interest to improve their removal during HD. For this, we need to understand the full picture of PBUT kinetics in the patient and the extracorporeal circuit.

The general aim of this thesis was therefore to obtain more insight into the kinetics of PBUTs and protein-bound antibiotics in patients with CKD. These kinetics were explored either *in vivo* and/or in different *in vitro* settings after development and/or optimization of analytical techniques.

The basic principles of the most important analytical methods used during the thesis are described in Chapter 2, while other techniques are described in the chapter of the corresponding study.

In Chapter 3, the *in house* developed ultra-high performance liquid chromatography method with ultraviolet and fluorescence detection (UHPLC-UV/FLD) for PBUT quantification was fine-tuned by checking the effect of sample temperature, pH, matrix and a single freeze/thaw

cycle on the %PB of PBUTs, using blood from HD patients. Furthermore, a novel ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS) assay was developed and validated for the quantification of the antibiotic teicoplanin.

In an *in vivo* study, provided in Chapter 4, PBUT %PB data was collected in 95 patients with different stages of CKD and 10 patients on hemodialysis. In these HD patients, kinetics with changes in %PB of PBUTs were evaluated by blood sampling at different time points during an HD session from the arterial as well as venous blood line.

The binding characteristics of PBUTs and their related competition are described in Chapter 5. These were explored *in vitro* in serum from healthy subjects as well as from HD patients, to include possible differences in albumin and binding characteristics. In addition, the %PB of the two antibiotics vancomycin and teicoplanin was compared *in vitro* in plasma from healthy subjects as well as from HD patients and the possible competition with PBUTs was checked.

In Chapter 6, PBUT distribution in a direct accessible extra-plasmatic compartment, *i.e.* the erythrocytes, is illustrated in blood from stable HD patients. Information on their transport across the erythrocyte membrane is provided as well and was obtained by loading and unloading experiments of erythrocytes from healthy subjects and HD patients, respectively.

This thesis is closed (Chapter 7) with a general summary and critical interpretation of the findings as well as the broader relevance of the work and future perspectives.

1.6 References

1. Glassock RJ, Winearls C (2009) Ageing and the Glomerular Filtration Rate: Truths and Consequences. Trans Am Clin Climatol Assoc 120:419–428.

2. Musso CG, Oreopoulos G (2011) Aging and Physiological Changes of the Kidneys Including Changes in Glomerular. Nephron Physiol 119:1–5. doi: 10.1159/000328010

3. Molnar C, Gair J (2013) The Kidneys and Osmoregulatory Organs. In: Concepts Biol. https://opentextbc.ca/biology/chapter/22-2-the-kidneys-and-osmoregulatory-organs/.

4. Levey AS, Coresh J, Bolton K, Culleton B, Harvey KS, Ikizler AT (2002) K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis 39:S1-266.

5. Coresh J, Selvin E, Stevens LA, Manzi J, Eggers P, Lente F Van, Levey AS (2007) Prevalence of Chronic Kidney Disease in the United States. J Am Med Assoc 298:2038–2047.

6. Zhang Q, Rothenbacher D (2008) Prevalence of chronic kidney disease in population-based studies : Systematic review. BMC Public Health 8:117. doi: 10.1186/1471-2458-8-117

7. McCullough K, Sharma P, Ali T, Khan I, Smith WCS, Macleod A, Black C (2012) Measuring the population burden of chronic kidney disease: a systematic literature review of the estimated prevalence of impaired kidney function. Nephrol Dial Transplant 27:1812–1821. doi: 10.1093/ndt/gfr547

8. Grams ME, Juraschek SP, Selvin E, Foster MC, Inker LA, Eckfeldt JH (2013) Trends in the Prevalence of Reduced GFR in the United States : A Comparison of Creatinine- and Cystatin C Based Estimates. Am J Kidney Dis 62:253–260. doi: 10.1053/j.ajkd.2013.03.013

9. Brück K, Stel VS, Gambaro G, Hallan S, Völzke H, Ärnlöv J, Kastarinen M, Guessous I, Vinhas J, Stengel B, Brenner H, Chudek J, Romundstad S, Tomson C, Gonzalez AO, Bello AK, Ferrieres J, Palmieri L, Browne G, Capuano V, Biesen W Van, Zoccali C, Gansevoort R, Navis G, Rothenbacher D, Ferraro PM, Nitsch D, Wanner C, Jager KJ (2016) CKD Prevalence Varies across the European General Population. J Am Soc Nephrol 27:2135–2147. doi: 10.1681/ASN.2015050542

10. Levey AS, Jong PE De, Coresh J, Nahas M El, Astor BC, Matsushita K, Gansevoort RT, Kasiske BL, Eckardt K (2011) The definition, classification, and prognosis of chronic kidney disease: a KDIGO Controversies Conference report. Kidney Int 80:17–28. doi: 10.1038/ki.2010.483

11. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group (2013) KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney Int Suppl 3:1–150. doi: 10.1038/kisup.2012.72

12. Delanaye P (2012) How Measuring Glomerular Filtration Rate? Comparison of Reference Methods. In: Sahay M (ed) Basic Nephrol. Acute Kidney Inj. InTech, pp 21–60

13. Lamb EJ, Stevens PE (2014) Estimating and measuring glomerular filtration rate: methods of measurement and markers for estimation. Curr Opin Nephrol Hypertens 23:258–266. doi: 10.1097/01.mnh.0000444813.72626.88

14. Cockcroft DW, Gault MH (1976) Prediction of creatinine clearance from serum creatinine. Nephron 16:31–41. doi: 10.1159/000180580

15. Levey AS, Bosch JP, Lewis JB, Greene T (1999) A More Accurate Method To Estimate Glomerular Filtration Rate from Serum Creatinine: A New Prediction Equation. Ann Intern Med 130:461–470. doi: 10.7326/0003-4819-130-6-199903160-00002

16. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van Lente F (2006) Using Standardized Serum Creatinine Values in the Modification of Diet in Renal Disease Study Equation for Estimating Glomerular Filtration Rate. Ann Intern Med 145:247–254. doi: 10.7326/0003-4819-145-4-200608150-00004

17. Levey AS, Stevens LA, Schmid CH, Zhang YL, Iii AFC, Feldman HI, Kusek JW, Eggers P, Lente F Van, Greene T (2009) A New Equation to Estimate Glomerular Filtration Rate. Ann Intern Med 150:604–612. doi: 10.7326/0003-4819-150-9-200905050-00006

18. Health Jade Team Kidney Failure. https://healthjade.com/kidney-failure/. Accessed 21 Nov 2017

19. Baxter Company Baxter - dialyzers. https://www.baxter.com/products-expertise/renal-failure-treatments/hemodialysis-products/dialyzers.page. Accessed 21 Nov 2017

20. Nipro Company Nipro - dialyzers. http://www.nipro.co.jp/en/business/device/renal/dialyzer/. Accessed 21 Nov 2017

21. Fresenius Medical Care Company Fresenius - dialyzers. http://www.fmc-ag.dk/files/Filterbrochure.pdf. Accessed 21 Nov 2017

22. Gallery For & Gt Hemodialysis. http://2.bp.blogspot.com/-yKcI_pIu6dU/UBPrNCj7IfI/AAAAAAAAAhA/oRz6Mh8C9pA/s1600/Hemodialysis.png. Accessed 21 Nov 2017

23. Van Biesen W, Vanholder R, Schepers E, Glorieux G, Dhondt A, Eloot S (2017) The Place of Large Pore Membranes in Hemodialysis. Contrib Nephrol 191:168–177. doi: 10.1159/000479265

24. Wratten M Lou, Ghezzi PM (2007) Hemodiafiltration with Endogenous Reinfusion. Contrib Nephrol 158:94–102. doi: 10.1159/000107239

25. Meijers BK, Weber V, Bammens B, Dehaen W, Verbeke K, Falkenhagen D, Evenepoel P (2008) Removal of the uremic retention solute p-cresol using fractionated plasma separation and adsorption. Artif Organs 32:214–219. doi: 10.1111/j.1525-1594.2007.00525.x

26. Brettschneider F, Tölle M, Von der Giet M, Passlick-Deetjen J, Steppan S, Peter M, Jankowski V, Krause A, Köhne S, Zidek W, Jankowski J (2013) Removal of Protein-Bound, Hydrophobic Uremic Toxins by a Combined Fractionated Plasma Separation and Adsorption Technique. Artif Organs. doi: 10.1111/j.1525-1594.2012.01570.x

27. Meyer TW, Peattie JWT, Miller JD, Dinh DC, Recht NS, Walther JL, Hostetter TH (2007) Increasing the Clearance of Protein-Bound Solutes by Addition of a Sorbent to the Dialysate. J Am Soc Nephrol 18:868–874. doi: 10.1681/ASN.2006080863

28. Sandeman SR, Howell CA, Phillips GJ, Zheng Y, Standen G, Pletzenauer R, Davenport A, Basnayake K, Boyd O, Holt S, Mikhalovsky S V. (2014) An adsorbent monolith device to augment the removal of uraemic toxins during haemodialysis. J Mater Sci Mater Med 25:1589–1597. doi: 10.1007/s10856-014-5173-9

29. Pavlenko D, van Geffen E, van Steenbergen MJ, Glorieux G, Vanholder R, Gerritsen KGF, Stamatialis D (2016) New low-flux mixed matrix membranes that offer superior

removal of protein-bound toxins from human plasma. Sci Rep 6:34429. doi: 10.1038/srep34429

30. Eloot S, Van Biesen W, Dhondt A, Billiet E, Verdonck P, Vanholder R (2008) Evidence for internal filtration in the Genius (R) system, performing slow low efficient daily dialysis in the intensive care unit. Blood Purif 26:460–467. doi: 10.1159/000157323

31. Meert N, Eloot S, Waterloos MA, Van Landschoot M, Dhondt A, Glorieux G, Ledebo I, Vanholder R (2009) Effective removal of protein-bound uraemic solutes by different convective strategies: A prospective trial. Nephrol Dial Transplant 24:562–570. doi: 10.1093/ndt/gfn522

32. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clarck W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jörres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W (2003) Review on uremic toxins: Classification, concentration and interindividual variability. Am J Soc Nephrol 63:1934–1943. doi: 10.1046/j.1523-1755.2003.00924.x

33. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A (2012) Normal and Pathologic Concentrations of Uremic Toxins. J Am Soc Nephrol 23:1258–1270. doi: 10.1681/ASN.2011121175

34. Neirynck N, Vanholder R, Schepers E, Eloot S, Pletinck A, Glorieux G (2013) An update on uremic toxins. Int Urol Nephrol 45:139–150. doi: 10.1007/s11255-012-0258-1

35. Vanholder R, Pletinck A, Schepers E, Glorieux G (2018) Biochemical and Clinical Impact of Organic Uremic Retention Solutes: A Comprehensive Update. Toxins (Basel) 10:33. doi: 10.3390/toxins10010033

36. Schepers E, Glorieux G, Vanholder R (2010) The Gut: The Forgotten Organ in Uremia? Blood Purif 29:130–136. doi: 10.1159/000245639

37. Aronov PA, Luo FJ, Plummer NS, Quan Z, Holmes S, Hostetter TH, Meyer TW (2011) Colonic Contribution to Uremic Solutes. J Am Soc Nephrol 22:1769–1776. doi: 10.1681/ASN.2010121220

38. Gryp T, Vanholder R, Vaneechoutte M, Glorieux G (2017) p-cresyl sulfate. Toxins (Basel). doi: 10.3390/toxins9020052

39. Hubbard TD, Murray IA, Perdew GH (2015) Special Section on Drug Metabolism and the Microbiome — Minireview Indole and Tryptophan Metabolism : Endogenous and Dietary Routes to Ah Receptor Activation. Drug Metab Dispos 43:1522–1535.

40. Leong S, Sirich T (2016) Indoxyl Sulfate—Review of Toxicity and Therapeutic Strategies. Toxins (Basel) 8:358. doi: 10.3390/toxins8120358

41. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 106:3698–3703. doi: 10.1073/pnas.0812874106

42. Mishima E, Fukuda S, Mukawa C, Yuri A, Kanemitsu Y, Matsumoto Y, Akiyama Y, Fukuda NN, Tsukamoto H, Asaji K, Shima H, Kikuchi K, Suzuki C, Suzuki T, Tomioka Y, Soga T (2017) Evaluation of the impact of gut microbiota on uremic solute accumulation by a CE-TOFMS-based metabolomics approach. Kidney Int 92:634–645. doi: 10.1016/j.kint.2017.02.011

43. Pero RW (2010) Health Consequences of Catabolic Synthesis of Hippuric Acid in Humans. Curr Clin Pharmacol 5:67–73. doi: 10.2174/157488410790410588

44. Itoh Y, Ezawa A, Kikuchi K, Tsuruta Y, Niwa T (2012) Protein-bound uremic toxins in hemodialysis patients measured by liquid chromatography/tandem mass spectrometry and their effects on endothelial ROS production. Anal Bioanal Chem 403:1841–1850. doi: 10.1007/s00216-012-5929-3

45. Masereeuw R, Mutsaers H, Toyohara T, Abe T, Jhawar S, Sweet DH, Lowenstein J (2014) The Kidney and Uremic Toxin Removal: Glomerulus or Tubule? Semin Nephrol 34:191–208. doi: 10.1016/j.semnephrol.2014.02.010

46. Viaene L, Annaert P, De Loor H, Poesen R, Evenepoel P, Meijers B (2013) Albumin is the main plasma binding protein for indoxyl sulfate and p-cresyl sulfate. Biopharm Drug Dispos 34:165–175. doi: 10.1002/bdd.1834

47. Smith D, Di L, Kerns E (2010) The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. Nat Rev 9:929–940. doi: 10.1038/nrd3287

48. Klammt S, Wojak HJ, Mitzner A, Koball S, Rychly J, Reisinger EC, Mitzner S (2012) Albumin-binding capacity (ABiC) is reduced in patients with chronic kidney disease

along with an accumulation of protein-bound uraemic toxins. Nephrol Dial Transplant 27:2377–2383. doi: 10.1093/ndt/gfr616

49. Bertuzzi A, Mingrone G, Gandolfi A, Greco A V., Ringoir S, Vanholder R (1997) Binding of indole-3-acetic acid to human serum albumin and competition with L-tryptophan. Clin Chim Acta 265:183–192.

50. Mingrone G, De Smet R, Greco AV, Bertuzzi A, Gandolfi A, Ringoir S, Vanholder R (1997) Serum uremic toxins from patients with chronic renal failure displace the binding of L-tryptophan to human serum albumin. Clin Chim Acta 260:27–34. doi: 10.1016/S0009-8981(96)06504-7

51. Bergé-Lefranc D, Chaspoul F, Cérini C, Brunet P, Gallice P (2013) Thermodynamic study of indoxylsulfate interaction with human serum albumin and competitive binding with p-cresylsulfate. J Therm Anal Calorim 115:2021–2026. doi: 10.1007/s10973-013-3067-6

52. Perucca E (1980) Plasma Protein Binding of Phenytoin in Health and Disease: Relevance to Therapeutic Drug Monitoring. Ther Drug Monit 2:331–344.

53. Vanholder R, Van Landschoot N, De Smet R, Schoots A, Ringoir S (1988) Drug protein binding in chronic renal failure: evaluation of nine drugs. Kidney Int 33:996–1004. doi: 10.1038/ki.1988.99

54. Mabuchi H, Nakahashi H (1988) Displacement by Anionic Drugs of Endogenous Ligands Bound to Albumin in Uremic Serum. Ther Drug Monit 10:261–264.

55. Takamura N, Maruyama T, Otagiri M (1997) Effects of uremic toxins and fatty acids on serum protein binding of furosemide: Possible mechanism of the binding defect in uremia. Clin Chem 43:2274–2280.

56. Davilas A, Koupparis M, Macheras P, Valsami G (2006) In-vitro study on the competitive binding of diflunisal and uraemic toxins to serum albumin and human plasma using a potentiometric ion-probe technique. J Pharm Pharmacol 58:1467–1474. doi: 10.1211/jpp.58.11.0007

57. Dreisbach AW, Lertora JJ (2008) The Effect of Chronic Renal Failure on Drug Metabolism and Transport. Expert Opin Drug Metab Toxicol 4:1065–1074. doi: 10.1517/17425255.4.8.1065

58. Johannessen Landmark C, Johannessen SI, Tomson T (2012) Host factors affecting antiepileptic drug delivery-Pharmacokinetic variability. Adv Drug Deliv Rev 64:896–910. doi: 10.1016/j.addr.2011.10.003

59. Zaidi N, Ahmad E, Rehan M, Rabbani G, Ajmal MR, Zaidi Y, Subbarao N, Khan RH (2013) Biophysical Insight into Furosemide Binding to Human Serum Albumin : A Study To Unveil Its Impaired Albumin Binding in Uremia. J Phys Chem B 117:2595–2604. doi: 10.1021/jp3069877

60. Tao X, Thijssen S, Kotanko P, Ho C, Henrie M, Stroup E, Handelman G (2016) Improved dialytic removal of protein-bound uraemic toxins with use of albumin binding competitors : an in vitro human whole blood study. Nat Publ Gr 2–10. doi: 10.1038/srep23389

61. Benet LZ, Hoener BA (2002) Changes in plasma protein binding have little clinical relevance. Clin Pharmacol Ther 71:115–121. doi: 10.1067/mcp.2002.121829

62. Spalding EM, Chamney PW, Farrington K (2002) Phosphate kinetics during hemodialysis: Evidence for biphasic regulation. Kidney Int 61:655–667. doi: 10.1046/j.1523-1755.2002.00146.x

63. Agar BU, Akonur A, Lo YC, Cheung AK, Leypoldt JK (2011) Kinetic model of phosphorus mobilization during and after short and conventional hemodialysis. Clin J Am Soc Nephrol 6:2854–2860. doi: 10.2215/CJN.03860411

64. Agar BU, Akonur A, Cheung AK, Leypoldt JK (2011) A simple method to estimate phosphorus mobilization in hemodialysis using only predialytic and postdialytic blood samples. Hemodial Int 15:9–14. doi: 10.1111/j.1542-4758.2011.00596.x

65. Eloot S, Van Biesen W, Vanholder R (2012) A sad but forgotten truth: The story of slow-moving solutes in fast hemodialysis. Semin Dial 25:505–509. doi: 10.1111/j.1525-139X.2012.01107.x

66. Eloot S, Schneditz D, Vanholder R (2012) What can the dialysis physician learn from kinetic modelling beyond Kt/Vurea? Nephrol Dial Transplant 27:4021–4029. doi: 10.1093/ndt/gfs367

67. Vanholder R, Dhondt A, Van Biesen W (2001) Is Kt/V Urea a Satisfactory Measure for Dosing the Newer Dialysis Regimens? Semin Dial 14:12–14. doi: 10.1046/j.1525-139x.2001.00003-3.x

68. Vanholder R, De Smet R, Lesaffer G (2002) Dissociation between dialysis adequacy and Kt/V. Semin Dial 15:3–7. doi: 10.1046/j.1525-139x.2002.00005.x

69. Yavuz A, Tetta C, Ersoy FF, D'Intini V, Ratanarat R, De Cal M, Bonello M, Bordoni V, Salvatori G, Andrikos E, Yakupoglu G, Levin NW, Ronco C (2005) Uremic toxins: A new focus on an old subject. Semin Dial 18:203–211. doi: 10.1111/j.1525-139X.2005.18313.x

70. Eloot S, Van Biesen W, Dhondt A, Van de Wynkele H, Glorieux G, Verdonck P, Vanholder R (2008) Impact of hemodialysis duration on the removal of uremic retention solutes. Kidney Int 73:765–770. doi: 10.1038/sj.ki.5002750

71. Eloot S, Schneditz D, Cornelis T, Van Biesen W, Glorieux G, Dhondt A, Kooman J, Vanholder R (2016) Protein-bound uremic toxin profiling as a tool to optimize hemodialysis. PLoS One 11:1–18. doi: 10.1371/journal.pone.0147159

Chapter 2

Analytical methods for the quantification of protein-bound uremic toxins and antibiotics

2.1 Introduction

In this thesis, free and total concentrations of protein-bound uremic toxins (PBUTs) and the protein-bound antibiotic agent vancomycin were determined by different validated analytical methods [1–4]. The method to quantify free and total teicoplanin concentrations has been further developed and validated during the course of the present thesis.

PBUT quantifications were performed by three different reversed-phase (ultra-)high performance liquid chromatography methods in which the compounds were detected either by ultraviolet or fluorescence detection [(U)HPLC – UV/FLD] [1,2] or by tandem mass spectrometry (UHPLC – MS/MS) [3]. Vancomycin was determined by a chemiluminescent microparticle immunoassay (CMIA) [4], whereas teicoplanin was quantified by reversed-phase UHPLC coupled to high resolution mass spectrometry (UHPLC-HRMS).

This chapter provides details and principles of the used methods.

2.2 Reversed-phase (ultra-)high performance liquid chromatography

2.2.1 Principle

(U)HPLC is a technique to separate, identify and quantify analytes in solution. In brief, degassed solvent (*i.e.* mobile phase) is continuously pumped through a (U)HPLC column, which can be temperature-controlled, under (ultra-)high pressure (typical < 400 bar in HPLC and 300 – 1200 bar in UHPLC). Typical flow rates in HPLC and UHPLC are 1 – 3 mL/min and 0.3 – 0.6 mL /min, respectively. In reversed-phase (U)HPLC, the mobile phase is mainly hydrophilic and can be constant (*i.e.* isocratic) or varied (*i.e.* gradient) in composition during the analysis. A reversed-phase column is packed with silica particles of which silanol groups are partly substituted by alkyl chains, where octadecylsilane (C-18, ODS), octylsilane (C-8) and phenyl(hexyl)silane are the most common ones.

2.2.2 Sample preparation

In general, most samples need to be pretreated prior to (U)HPLC analysis. Amongst others, sample preparation might be necessary to concentrate, purify, extract or derivatize the analytes to enhance the sensitivity or for column lifetime issues. In this thesis, serum and plasma samples were analyzed and needed a pretreatment step to precipitate serum or plasma proteins to prevent column clogging. Proteins can e.g. be precipitated by heating the sample, or by adding organic solvents such as methanol or acetonitrile.

During the sample preparation, preferably at the start, a known amount of internal standard can be added to both the samples and calibrators (see 2.2.4.4 Quantification). In this way, it is possible to correct for analyte losses during sample preparation or for a variation in instrument injection. Ideally, the internal standard is a compound that is not present in the native samples, stable during sample preparation, well-resolved during the analysis and very similar in molecular structure as compared to the analyte. In some methods, more than one internal standard can be added. This is especially the case when the analytes are detected by mass spectrometry. Here, the deuterated form of preferably each analyte is added to the sample, when available and affordable. These deuterated forms behave similarly as their non-deuterated forms but are detected at a different mass-to-charge ratio (see 2.2.4.3 Mass spectrometry) and are therefore the ideal internal standards.

In most of the presented work, both total and free (*i.e.* unbound) serum or plasma analyte concentrations were determined. Free concentrations were obtained either indirectly by using equilibrium dialysis or directly by means of ultrafiltration.

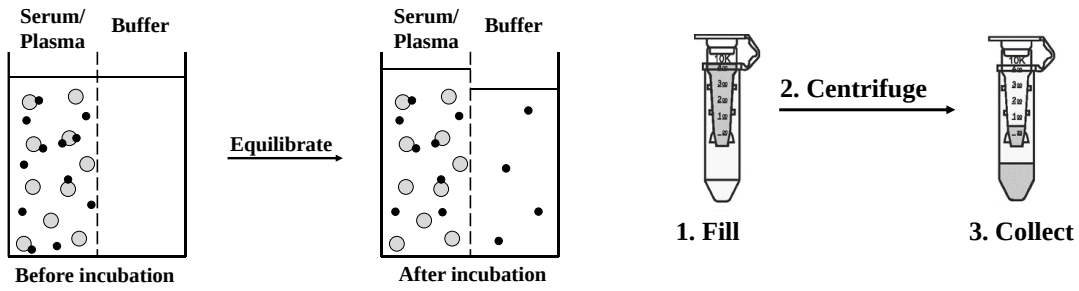
Equilibrium dialysis (ED) is the most widely used method to obtain free analyte fractions and is often seen as the gold standard [5,6]. This method is based on the equilibration of free fractions over two chambers which are separated by a semi-permeable membrane (Figure 2-1 left). At the start, one chamber is filled with serum or plasma while (ideally) an equal volume of buffer is added to the other chamber. Subsequently, free analytes equilibrate over the total (*i.e.* plasma + buffer) volume and cause the release of bound fraction. After several hours, typically 5 – 6 hours, a new equilibrium is established and analytes can be quantified in both serum/plasma and buffer chambers. Finally, the free analyte fractions are obtained by dividing the analyte concentrations on the buffer side by the analyte concentrations on the serum/plasma side. The original free analyte concentrations in the sample are subsequently obtained by multiplying the free fractions by the original total analyte concentrations in the sample. The time needed for equilibration can be determined in a pilot experiment in which samples are taken at different time points.

These chambers need to be sealed during dialysis to prevent shifts in pH, as this might induce a shift in free fraction [7–9]. Furthermore, due to serum or plasma oncotic pressure, free fractions may be higher than in the original sample [5,6,10,11]. The Donnan effect may arise due to the impermeability of the membrane for large charged plasma proteins, and can result in the shift of charged free compounds towards the buffer chamber [5]. However, shifts in free fractions due to oncotic pressure or the Donnan effect can be reduced to a minimum by using isotonic buffers, such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or phosphate buffered saline (PBS) [5]. Moreover, non-specific binding to the ED device and membrane needs to be studied when recovery is important [5,9].

Ultrafiltration (UF) is an alternative for ED and is characterized by a much higher analysis speed, which is typically 10 – 30 minutes. In UF, serum or plasma containing protein-bound analytes is ultrafiltered during centrifugation through the pores of a semi-permeable membrane. This process results in fluid containing free analytes (*i.e.* ultrafiltrate) at the other side of the membrane (Figure 2-1 right). Finally, the free concentration can be directly determined in the ultrafiltrate. Also here, non-specific binding and the Donnan effect may result in inaccurate free concentrations. It is debatable whether the continuous removal of free compound during

centrifugation can cause a shift in free fraction and consequently result in inaccurate free concentrations as well [5,6].

Figure 2-1 Left: Principle of equilibrium dialysis for an analyte with a free fraction of 50 %. Right: Principle of ultrafiltration, based on the use of Merck Amicon filters [12].



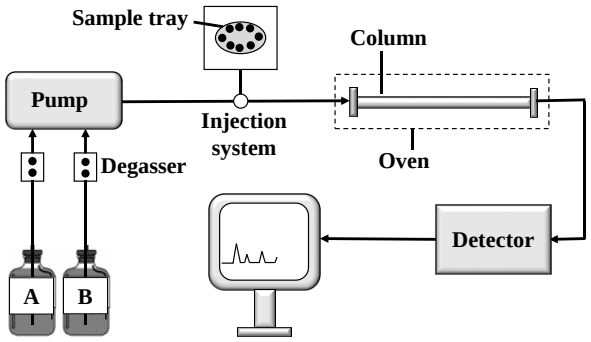
In this thesis, both ED and UF were used to obtain free analyte concentrations. In ED, PBS buffer and ultrafiltrate obtained from healthy serum were used. A small volume shift due to oncotic pressure was observed and concentrations were corrected for this shift. In addition, the possible non-specific binding to ED or UF membranes was studied as well. Both will be discussed further in this thesis.

2.2.3 From injection to detection

After sample preparation, samples are transferred into autosampler vials and placed in a sample tray, which can be temperature-controlled (Figure 2-2). When the sample is injected (typically 30 – 50 µL in HPLC and 1 – 30 µL in UHPLC), analytes travel with the mobile phase through the column and interact with the hydrophobic stationary phase (e.g. C18, C8). The more hydrophilic analytes show no or only a small affinity for the stationary phase and are not or minimally retained by the column. Hence, these more hydrophilic analytes will elute first and will be subsequently detected first. Depending on the analytes present in the injected sample, different interactions between analytes and stationary phase will result in different elution orders. As already mentioned before, the composition of the mobile phase can be modified during a run. This is typically performed to ensure that very hydrophobic analytes elute as well. For example, the mobile phase can consist of 95 % aqueous buffer (solvent A) and 5 % organic solvent (solvent B, e.g. methanol, acetonitrile) at the start of the analysis and change to 10 % buffer and 90 % organic solvent at the end. From the column, every eluted analyte travels with the mobile phase towards the detector.

UHPLC differs from HPLC by its shorter columns (typical 50 – 100 mm in UHPLC *versus* 100 – 300 mm in HPLC), smaller column internal diameters (typical 2.1 mm in UHPLC *versus* 4.6 mm in HPLC) and smaller particles (typical 1.7 – 2.6 µm in UHPLC *versus* 3.5 – 5 µm in HPLC). This results in a better resolution, higher sensitivity, faster analyses and less solvent consumption in UHPLC as compared to HPLC [13,14].

Figure 2-2 Simplified schematic representation of an (U)HPLC system.



2.2.4 Detection mechanisms

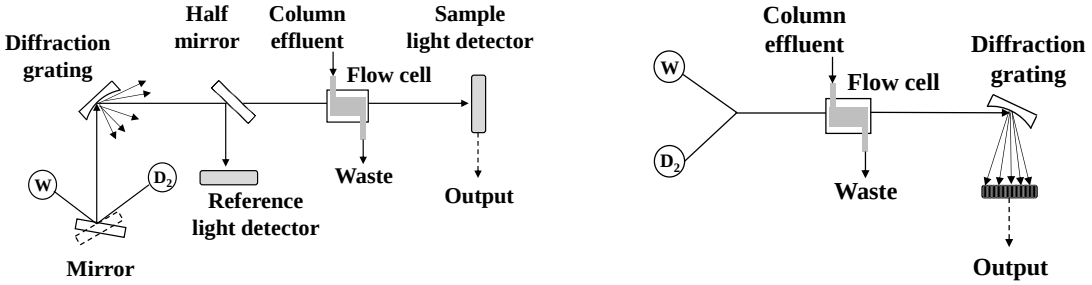
Analytes eluting from the column are further transported by the mobile phase towards the detector. Only detector systems used during the thesis will be discussed in this section.

2.2.4.1 Diode array detector

In a traditional ultraviolet (UV) or UV – visible light (UV – VIS) detector, a deuterium (λ = 190 – 380 nm) or tungsten (λ = 380 – 900 nm) lamp is used as light source, respectively. Subsequently, the emitted light is diffracted and dispersed, and one specific wavelength is guided to the flow cell (Figure 2-3 left). The mobile phase flows continuously through this cell and a difference in absorption is observed when an eluted analyte passes through the cell. Only one specific wavelength can be registered in a traditional UV detector.

In a diode array detector (DAD), also known as photodiode array detector (PDA), light emitted from the lamp is guided directly to the flow cell and is diffracted and dispersed after passing through the cell (Figure 2-3 right). Because of the multiple diode arrays in this detector, multiple wavelengths can be registered simultaneously.

Figure 2-3 Simplified schematic illustration of a traditional UV(-VIS) detector (left) and a diode array detector (right).

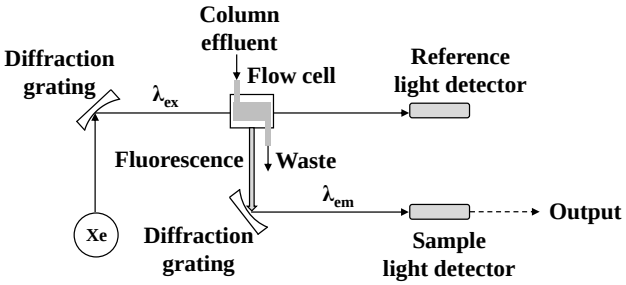


D: deuterium lamp; W: tungsten lamp. Adapted from [15].

2.2.4.2 Fluorescence detector

Some compounds have the characteristic to emit light at one specific wavelength (λ_{em}) after being excited by another specific wavelength (λ_{ex}), where $\lambda_{ex} < \lambda_{em}$. This process is called fluorescence and is, besides UV detection, also commonly used in (U)HPLC analyses. In fluorescence detection, a xenon lamp can be used as light source and the emitted light is diffracted and dispersed to a specific excitation wavelength (Figure 2-4). This light travels through the flow cell and a passing analyte can be excited, followed by its relaxation by the emission of light with a lower energy. This emitted light is then, orthogonal to the exciting light, guided towards the detector.

Figure 2-4 Simplified schematic illustration of a fluorescence detector.



Xe: xenon lamp. Adapted from [16].

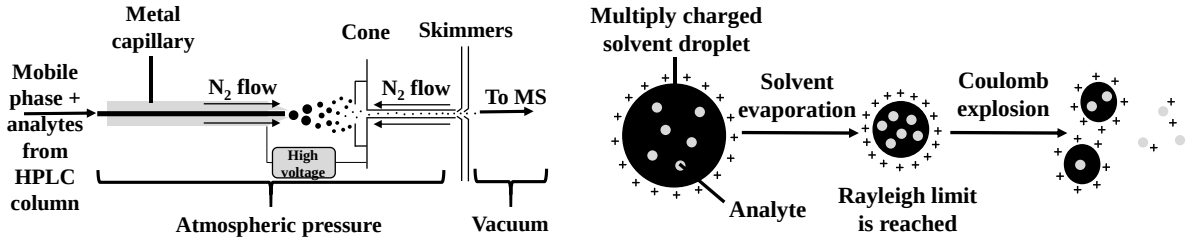
2.2.4.3 Mass spectrometry

Mass spectrometry (MS) is a technique in which gaseous ions are separated based on their mass-to-charge (m/z) ratio. Besides providing information on molecular weight, mass spectrometers are also able to elucidate the molecular structure of an analyte. In general, a mass spectrometer is composed of an ion source, mass analyzer and detector. In the ion source,

compounds eluting from the column are ionized and transferred into the gaseous phase and subsequently guided to the mass analyzer, which operates under high vacuum. Here, the ions are separated based on their m/z ratio by applying a magnetic and/or electric field. Finally, the ion beam is transferred into a signal in the detector. Various types of ion sources and mass analyzers are available but only those used during the thesis will be discussed in this paragraph.

(Heated) electrospray ionization (ESI) is a soft ionization technique, which means that the ionization results in only a limited amount of fragmentation. The mobile phase containing analytes is guided into a (heated) metal capillary with a fine tip, under a high positive or negative voltage (typically 2.5 – 6.0 kV). A nitrogen flow is applied to nebulize the flow of mobile phase and relatively large charged droplets are formed (Figure 2-5 left). Depending on the polarity of the applied voltage, these droplets can be positively or negatively charged. In positive mode (ESI+), predominantly protonated compounds are formed which results in peaks at $[MW+n]^{n+}$. In negative mode (ESI-), predominantly deprotonated compounds are formed which results in peaks at $[MW-n]^{n-}$. Usually, n equals 1 in both positive and negative modes. Due to an excess of droplets having the same charge, these droplets are repulsed resulting in a so-called conical electrospray (Figure 2-5 left). Subsequently, the mobile phase is evaporated. This process is enhanced by applying a drying gas, such as nitrogen, in an opposite direction. As a result, the droplet size decreases and at a certain moment, the electrostatic repulsion between ions with the same charge becomes higher than the surface tension of the droplet, *i.e.* the Rayleigh limit. At this moment, the droplets explode (*i.e.* Coulomb explosion) into smaller droplets with a lower surface charge density. The process of decreasing in size and exploding into smaller droplets is repeated and finally, single analyte ions in the gaseous phase are formed (right). These ions are then guided from the ESI source, which operates at atmospheric pressure, towards the mass analyzer which operates at high vacuum [17].

Figure 2-5 Principle of electrospray ionization.



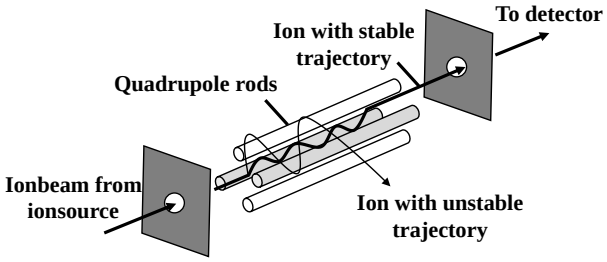
Adapted from [18].

Two different mass analyzers were used in this thesis, *i.e.* a tandem mass spectrometer (MS/MS) and a high resolution mass spectrometer (HRMS).

A tandem quadrupole mass spectrometer is typically composed of three quadrupole mass analyzers, positioned in a linear way and is therefore often called 'triple quadrupole'.

In general, a quadrupole mass analyzer consists of four parallel metal rods and each opposing rod is connected together electrically (Figure 2-6). A specific electrical field is formed by applying a radiofrequency (RF) and direct current (DC) voltage to these rods. Analyte ions travel through the quadrupole and only those with a specific m/z ratio have a stable trajectory at the selected electrical field and are guided to the detector. All other ions have unstable trajectories and are removed. Changing the electrical field allows ions with other m/z ratios to travel through the quadrupole followed by their detection.

Figure 2-6 Simplified schematic illustration of a quadrupole mass analyzer.



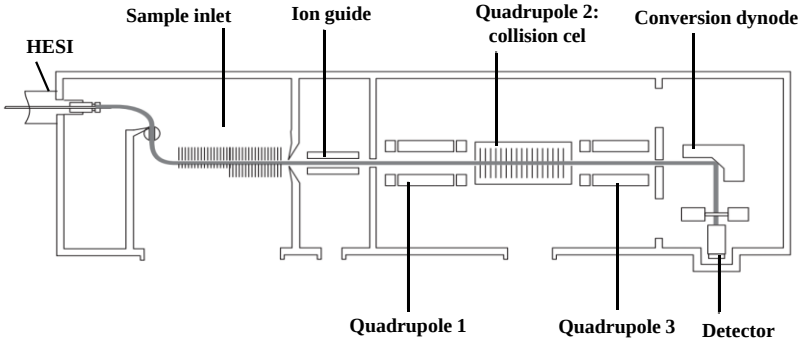
Adapted from [19].

Analyte ions which are formed by (H)ESI show a low degree of fragmentation and the resulting spectra mainly contain the molecular ions. In some cases it might be desirable to create fragments of the analyte ions, e.g. in molecular structure elucidation or when ions with the same m/z ratio interfere with the analyte ion of interest. This fragmentation can be achieved by using a triple quadrupole system (Figure 2-7). The analyte ion of interest is selected in the first quadrupole (Q1) and allowed to collide with a collision gas (typical argon) to form fragments. This fragmentation occurs in a collision cell which is usually an RF-only quadrupole (Q2) or hexapole. In the third quadrupole (Q3), specific fragments can be selected to travel towards the detector. In this thesis, a photomultiplier was used to detect ions that were selected in Q3 [20,21]. In this type of detector, an ion hits a conversion dynode and results in the emission of electrons. These electrons are accelerated towards a scintillation substance which on its turn emits photons after the electrons collide to this substance. The emitted photons collide to a photocathode and electrons are ejected from the photocathode surface as a consequence of the

photoelectric effect. On their way to the anode, these ejected electrons are multiplied and finally, the large number of electrons reaching the anode produce an electric signal.

Accordingly, it is possible to e.g. select an analyte ion of interest in Q1 and select a specific fragment of that analyte in Q3. In this way, two or more ions with the same m/z ratio that co-elute from the (U)HPLC column can be separated, resulting in a much higher sensitivity. This mode is called multiple reaction monitoring (MRM) and is commonly used in MS/MS analyses.

Figure 2-7 Schematic representation of the tandem mass spectrometer used in this thesis (Xevo TQS, Waters).



ESI: electrospray ionization. Adapted from [20].

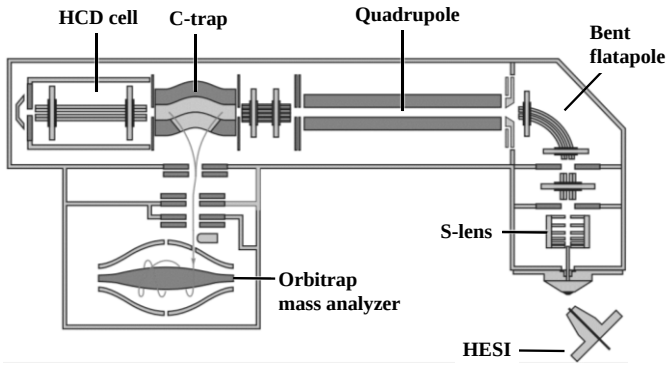
In a quadrupole, ions are selected based on their nominal mass. Consequently, different ions with the same nominal mass and charge, but a different exact mass, have the same m/z ratio and cannot be separated by a single quadrupole. Therefore, a triple quadrupole can be used for the detection of only e.g. fragment ions of the specific analyte of interest. Alternatively, a high resolution mass spectrometer (HRMS) is capable to resolve ions with the same nominal mass, but a different exact mass. This type of mass spectrometer is characterized by its high resolution and high mass accuracy.

In the HRMS method used in this research, analyte ions are formed by heated ESI followed by the introduction into the mass spectrometer (Figure 2-8). These ions are further focused by a stacked ring ion guide (S-lens) and residual neutrals are removed using the bent flatapole. In a next step, ions with a specific nominal m/z ratio can be selected via the quadrupole prior to be accumulated in the C-trap. Subsequently, analyte ions are injected into the orbitrap in which ions with different exact masses are separated. The orbitrap is composed of a central electrode surrounded by two outer electrodes, which enable it to function as both a mass analyzer and detector. When a packet of analyte ions is injected from the C-trap into the orbitrap, they

oscillate around the central electrode and in between the two outer electrodes. Finally, analyte ions with different exact masses are separated because of their different axial and spatial oscillation frequencies.

The frequencies of the axial oscillations are measured on the outer electrodes by the process of image current detection. Optionally, analyte ions can be fragmented in the high energy collision dissociation cell (HCD cell), before being trapped in the C-trap and injected into the orbitrap [22,23].

Figure 2-8 Schematic representation of the high resolution mass spectrometer used in this thesis (Q-Exactive Plus, Thermo Fisher Scientific).



HESI: heated electrospray ionization; HCD: high energy collision dissociation. Adapted from [23].

2.2.4.4 Quantification

In (U)HPLC analyses, analytes can be quantified by external calibration. In this calibration method, a series of calibrator standards are prepared, containing an increasing known amount of the analytes of interest. These calibrator standards may contain the same amount of internal standard as the samples and a calibration curve is obtained by plotting the analyte-to-internal standard area ratios to their amount ratios in the different calibrator levels. This calibration curve can be linear, quadratic or exponential, with different weightings, e.g. equal weighting, $1/x$ and $1/x^2$. Quantification of the unknown analytes is performed by relating their analyte-to-internal standard area ratio to the calibration curve.

2.2.5 Detailed summary of used (U)HPLC methods

Depending on the date of analysis and/or the collaboration with other laboratories, different (U)HPLC methods were used for the quantification of PBUTs. In the *in vivo* study as described in Chapter 4, PBUTs were determined by HPLC-UV/FLD in which two different columns were

used: one for the separation of hippuric acid, indole-3-acetic acid and indoxyl sulfate [1] and one for the separation of *p*-cresylglucuronide and *p*-cresyl sulfate [2]. More recent studies included the analysis of PBUTs by UHPLC-UV/FLD (Chapters 3, 5 and 6) or UHPLC tandem MS [3] (Chapter 5), both using a single column for the separation of the above listed compounds.

Teicoplanin (*i.e.* compounds A3-1, A2-1, A2-2 & A2-3 and A2-4 & A2-5) was quantified by UHPLC-HRMS (Chapters 3 and 6). The used chromatographic settings are summarized in Table 2-1 and the used UV/FLD and mass spectrometer settings in Table 2-2 and Table 2-3, respectively.

2.3 Chemiluminescent microparticle immunoassay

For the quantification of free and total vancomycin, a chemiluminescent microparticle immunoassay (CMIA) method was used [4]. This technique is based on the detection of light, which is emitted following a chemical reaction.

This CMIA method mixes sample containing vancomycin, anti-vancomycin paramagnetic microparticles and vancomycin acridinium-labeled conjugates (Figure 2-9). The anti-vancomycin coated microparticles bind to vancomycin present in the sample and to the vancomycin acridinium-labeled conjugates. Subsequently, a magnet attracts the paramagnetic microparticles to the wall and a washing step with PBS removes all compounds that are not bound to the microparticles. After a second washing step, a so-called pre-trigger (1.32 w/v % H_2O_2) is added and the background signal is recorded. The created acid milieu pre-activates the acridinium. In a next step, a trigger solution (0.35 M NaOH) is added and an oxidative chemiluminescent reaction leads to the formation of N-methylacridone. Emitted photons travel towards the detector, which is a photomultiplier, and strike the photocathode. Electrons are ejected from the surface of the photocathode, multiplied and finally generate an electrical signal, which is expressed as relative light units (RLUs). These RLUs are inversely correlated to the amount of vancomycin present in the sample.

The main benefit of this method is that sample preparation is not required. Hence, an autosampler vial can directly be filled with serum or plasma, making this method suitable for high-throughput routine analyses. Free vancomycin analysis, however, involves the separation of the free fraction by means of equilibrium dialysis or ultrafiltration prior to CMIA analysis [4].

Figure 2-9 Principle of the chemiluminescent microparticle immunoassay used for vancomycin quantification.

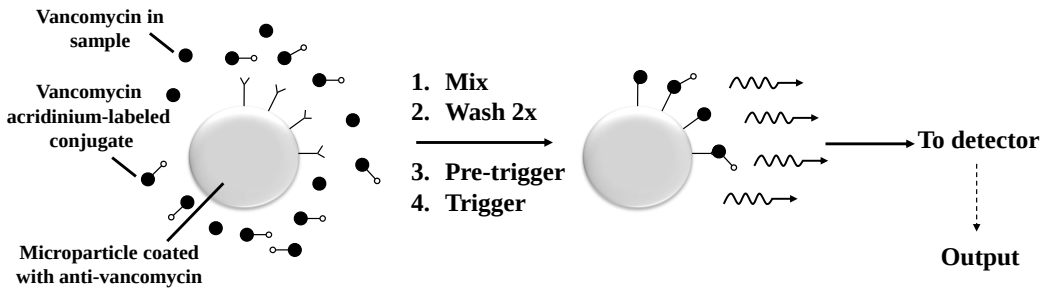


Table 2-1 Summary of used liquid chromatography settings for the analysis of protein-bound uremic toxins and teicoplanin.

Analytes	Lab	Instrument		Mobile Phase			Column						
		Brand	Assay	Composition (start)	Composition (before re-equilibration)	Flow rate (mL/min)	Brand	Injection volume (µL)	Stationary Phase	Length (mm)	Internal diameter (mm)	Particle diameter (µm)	Column temperature (° C)
HA IAA IS	UZGent Nephrology (2008-2011)	2690 Alliance (Waters)	HPLC – UV/FLD	$HCOONH_4$ (50 mM, pH 3) and MeOH 98:2	$HCOONH_4$ (50 mM, pH 3) and MeOH (B) 0:100	1.0	Ultrasphere ODS (Beckman Instruments)	30	C18	250	4.6	5	22
***p*CG *p*CS**	UZGent Nephrology (2008-2011)	2690 Alliance (Waters)	HPLC – FLD	$HCOONH_4$ (50 mM, pH 3) and MeOH 65:35	$HCOONH_4$ (50 mM, pH 3) and MeOH 30:70	1.0	Ultrasphere ODS (Beckman Instruments)	60	C18	150	4.6	5	RT
HA IAA IS *p*CS	UZGent Nephrology (2014-2018)	1290 Infinity (Agilent Technologies)	UHPLC – UV/FLD	$HCOONH_4$ (50 mM, pH 3) and MeOH 98:2	$HCOONH_4$ (50 mM, pH 3) and MeOH (B) 0:100	0.3	Acquity BEH (Waters)	18	C18	100	2.1	1.7	26
HA IAA IS *p*CS	UZLeuven Nephrology (2015-2016)	Acquity H Class + Xevo TQS (Waters)	UHPLC – MS/MS	HCOOH (0.1%) and MeOH 97:3	HCOOH (0.1%) and MeOH 5:95	0.5	Acquity CSH (Waters)	5	fluorophenyl	50	2.5	1.7	40
Teico-planin	UZGent Toxicology and Routine (2017-2018)	Q-Exactive Plus (Thermo Fisher Scientific)	UHPLC – HRMS	$HCOONH_4$ (2 mM) + 0.1 % HCOOH and $HCOONH_4$ (2mM) + 0.1 % HCOOH + MeOH + ACN (50:50) 75:25	$HCOONH_4$ (2 mM) + 0.1 % HCOOH and $HCOONH_4$ (2mM) + 0.1 % HCOOH + MeOH + ACN (50:50) 10:90	0.4	Thermo Accucore	1 and 2	phenylhexyl	100	2.1	2.6	40

*p*CG: *p*-cresyl glucuronide; HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate; (U)HPLC: (ultra-)high performance liquid chromatography; C18: octadodecyl; RT: room temperature.

Table 2-2 Summary of used UV and FLD settings for the analysis of protein-bound uremic toxins and teicoplanin.

Analytes	Lab	Instrument		Detector	
		Brand	Assay	Type	Selected wavelength (nm)
HA **IAA** **IS**	UZGent Nephrology (2008-2011)	2690 Alliance (Waters)	HPLC – UV/FLD	UV FLD FLD	254 λ_{ex}: 280, λ_{em}: 340 λ_{ex}: 280, λ_{em}: 340
***p*CG** ***p*CS**	UZGent Nephrology (2008-2011)	2690 Alliance (Waters)	HPLC – FLD	FLD FLD	λ_{ex}: 265, λ_{em}: 290 λ_{ex}: 265, λ_{em}: 290
HA **IAA** **IS** ***p*CS**	UZGent Nephrology (2014-2018)	1290 Infinity (Agilent Technologies)	UHPLC – UV/FLD	UV FLD FLD FLD	245 λ_{ex}: 280, λ_{em}: 350 λ_{ex}: 280, λ_{em}: 376 λ_{ex}: 264, λ_{em}: 290

HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate; (U)HPLC: (ultra-)high performance liquid chromatography; UV: ultraviolet; FLD: fluorescence detection.

Table 2-3 Summary of used mass spectrometry settings for the analysis of protein-bound uremic toxins and teicoplanin.

Analytes	Lab	Instrument		Ionization		Tandem MS		Orbitrap
		Brand	Assay	Ionization mode	Temperature (°C)	Parent ion m/z	Daughter ion m/z	Ion m/z
HA IAA IS *p*CS	UZLeuven Nephrology (2015-2016)	Acquity H Class + Xevo TQS (Waters)	UHPLC – MS/MS	HESI- HESI+ HESI- HESI-	150	178.1 176.2 212.1 187.1	134.1 130.1 80.1 107.0	n.a.
Teicoplanin A3-1 A2-1 A2-2 & A2-3 A2-4 & A2-5	UZGent Toxicology and Routine (2017-2018)	Q-Exactive Plus (Thermo Fisher Scientific)	UHPLC – HRMS	HESI+	300	n.a.	n.a.	782.183/782.685/783.183/783.683/784.184 938.778/939.280/939.779/940.280/940.780 939.786/940.287/940.786/941.286/941.786 946.793/947.295/947.794/948.295/948.794

HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate; UHPLC: ultra-high performance liquid chromatography; MS/MS: tandem mass spectrometry; HRMS: high resolution mass spectrometry; HESI + or -: heated electrospray ionization positive or negative mode, respectively; underlined ion m/z values are for total teicoplanin analysis whereas the sum of five ion m/z values was made for free teicoplanin analysis.

2.4 References

1. Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339–347. doi: 10.1053/ajkd.2002.34518

2. Meert N, Schepers E, Glorieux G, Van Landschoot M, Goeman JL, Waterloos MA, Dhondt A, Van Der Eycken J, Vanholder R (2012) Novel method for simultaneous determination of p-cresylsulphate and p-cresylglucuronide: Clinical data and pathophysiological implications. Nephrol Dial Transplant 27:2388–2396. doi: 10.1093/ndt/gfr672

3. de Loor H, Poesen R, De Leger W, Dehaen W, Augustijns P, Evenepoel P, Meijers B (2016) A liquid chromatography – tandem mass spectrometry method to measure a selected panel of uremic retention solutes derived from endogenous and colonic microbial metabolism. Anal Chim Acta 936:149–156. doi: 10.1016/j.aca.2016.06.057

4. Stove V, Coene L, Carlier M, Waele JJ De, Fiers T, Verstraete AG (2015) Measuring Unbound Versus Total Vancomycin Concentrations in Serum and Plasma : Methodological Issues and Relevance. Ther Drug Monit 37:180–187.

5. Banker MJ, Clark TH (2008) Plasma / Serum Protein Binding Determinations. Curr Drug Metab 9:854–859.

6. Vuignier K, Schappler J, Veuthey J-L, Carrupt P-A, Martel S (2010) Drug-protein binding: a critical review of analytical tools. Anal Bioanal Chem 398:53–66. doi: 10.1007/s00216-010-3737-1

7. Brørs O, Jacobsen S (1985) pH lability in serum during equilibrium dialysis. Br J Clin Pharmacol 20:85–88.

8. Kochansky CJ, McMasters DR, Lu P, Koeplinger K a., Kerr HH, Shou M, Korzekwa KR (2008) Impact of pH on plasma protein binding in equilibrium dialysis. Mol Pharm 5:438–448. doi: 10.1021/mp800004s

9. Kratzer A, Liebchen U, Schleibinger M, Kees MG, Kees F (2014) Determination of free vancomycin, ceftriaxone, cefazolin and ertapenem in plasma by ultrafiltration: Impact of experimental conditions. J Chromatogr B Anal Technol Biomed Life Sci 961:97–102. doi: 10.1016/j.jchromb.2014.05.021

10. Tozer TN, Gambertoglio JG, Furst DE, Avery DS, Holford NH (1983) Volume shifts and protein binding estimates using equilibrium dialysis: application to prednisolone binding in humans. J Pharm Sci 72:1442–1446.

11. Banker MJ, Clark TH, Williams JA (2003) Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. J Pharm Sci 92:967–974. doi: 10.1002/jps.10332

12. Merck Company Amicon Ultra-0.5mL Centrifugal Filters for DNA and Protein Purification and Concentration. http://www.merckmillipore.com/BE/en/product/Amicon-Ultra-0.5nbspmL-Centrifugal-Filters-for-DNA-and-Protein-Purification-and-Concentration,MM_NF-C82301?ReferrerURL=http%3A%2F%2Fwww.emdmillipore.com%2FUS%2Fen%2Fproduct%2FAmicon-Ultra-0.5nbspmL-Centrifugal-F. Accessed 19 Dec 2017

13. Waters Company (2007) Waters AAPS 2007 Seminars. http://www.waters.com/webassets/cms/library/docs/720002425en.pdf. Accessed 19 Dec 2017

14. Chawla G, Ranjan C (2016) Principle, Instrumentation, and Applications of UPLC: A Novel Technique of Liquid Chromatography. Open Chem J 3:1–16. doi: 10.2174/1874842201603010001

15. Hitachi High-Technologies GLOBAL 7. Principle and Feature of Various Detection Methods (1). https://www.hitachi-hightech.com/global/products/science/tech/ana/lc/basic/course7.html#jump2. Accessed 20 Dec 2017

16. Hitachi High-Technologies GLOBAL 8. Principle and Feature of Various Detection Methods (2). https://www.hitachi-hightech.com/global/products/science/tech/ana/lc/basic/course8.html#jump1. Accessed 20 Dec 2017

17. Bruins AP (1998) Mechanistic aspects of electrospray ionization. J Chromatogr A 794:345–357. doi: 10.1016/S0021-9673(97)01110-2

18. Gates P (2014) Electrospray Inisation (ESI). In: Univ. Bristol - Sch. Chem. - Mass Sepctrometry Facil. http://www.chm.bris.ac.uk/ms/esi-ionisation.xhtml. Accessed 20 Dec 2017

19. Gates P (2014) Quadrupole Mass Analysis. In: Univ. Bristol - Sch. Chem. - Mass Spectrom. Facil. http://www.chm.bris.ac.uk/ms/quadrupole.xhtml. Accessed 20 Dec 2017

20. Waters Corporation (2016) Waters Xevo TQ-S micro Overview and Maintenance Guide. http://www.waters.com/webassets/cms/support/docs/715004599rB.pdf. Accessed 21 Dec 2017

21. Waters Corporation (2017) Xevo TQS Instrument Specifications. http://www.waters.com/webassets/cms/library/docs/720003447en.pdf. Accessed 21 Dec 2017

22. Makarov A, Scigelova M (2010) Coupling liquid chromatography to Orbitrap mass spectrometry. J Chromatogr A 1217:3938–3945. doi: 10.1016/j.chroma.2010.02.022

23. Thermo Fisher Scientific (2012) Exactive Plus ™ Operating Manual.

Chapter 3

Development and optimization of analytical methods for the quantification of protein-bound uremic toxins and antibiotics

3.1 Introduction

Prior to analysis, serum and plasma samples need to be pretreated and an important step in this sample preparation is the separation of the free fraction. Equilibrium dialysis (ED) and ultrafiltration (UF) are the two most commonly used methods for this purpose. During ED and UF there are experimental parameters possibly affecting the percentage protein binding (%PB) of protein-bound uremic toxins (PBUTs).

In addition to previously validated methods for PBUT quantification, a new analytical method was developed and validated to quantify the total and free concentration of the antibiotic teicoplanin by ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS).

In this chapter, the possible effect of non-specific adsorption (NSA) of PBUTs to ED and UF membranes is discussed as well as the comparison of ED and UF to measure free PBUT concentrations. Furthermore, the possible effect of sample temperature, pH and matrix on the %PB of PBUTs is described. In a last section, the development and validation (including NSA testing and ED/UF comparison) of the analytical method to quantify total and free teicoplanin are provided.

3.2 Non-specific adsorption of protein-bound uremic toxins to equilibrium dialysis or ultrafiltration membranes

3.2.1 Introduction

In both ED and UF, a semi-permeable membrane is used to separate the free from the protein-bound fraction in serum and plasma samples. Typically, these membranes are made of regenerated cellulose and have a specific molecular weight cut-off (MWCO) ranging from 3.5 kDa to 100 kDa [1]. Analytes of interest may, however, be adsorbed to these membranes and can consequently lead to an underestimation of the free analyte fraction, and thus an overestimation of the %PB [2,3]. The influence of this NSA to ED and UF membranes on the %PB is even more pronounced for highly protein-bound analytes.

Therefore, the extent of NSA to ED and UF membranes should be studied prior to use the particular ED or UF method. When NSA is present, either a mathematical correction can be used or calibration standards can be dialyzed or filtered, together with the unknown samples.

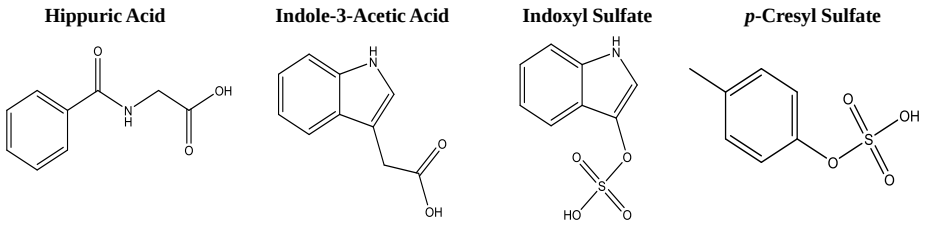
A small experiment was conducted to check whether the studied PBUTs show NSA to the ED and UF membranes used during this thesis.

3.2.2 Materials and methods

3.2.2.1 Chemicals

Hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA) and *p*-cresyl sulfate (*p*CS) was obtained from TCI Chemicals (Zwijndrecht, Belgium). Water (HPLC grade) was purchased from Acros Organics (Thermo Scientific, Geel, Belgium). The chemical structures of HA, IAA, IS and *p*CS are presented in Figure 3-1.

Figure 3-1 Chemical structures of the measured protein-bound uremic toxins.



3.2.2.2 Equilibrium dialysis and ultrafiltration

Equilibrium dialysis (n = 3). An HTDialysis 96b system (HTDialysis, Connecticut, USA) system was used. The dialysis membranes, consisting of regenerated cellulose with a MWCO of 12-14 kDa, were used according to the manufacturer's guidelines. A mixed stock solution with theoretical concentrations of 14 (HA), 1.4 (IAA), 5.2 (IS) and 7 (*p*CS) mg/L was prepared in PBS buffer and 150 µL was transferred into an ED chamber. The stock solution was dialyzed against PBS buffer (150 µL, pH = 7.4) for 5 h at room temperature (RT) on a reciprocating shaker. The wells were sealed with an adhesive film until equilibrium between the two chambers was established, *i.e.* after 5 h (determined in a pilot experiment, data not shown). Subsequently, the mixture of 4 PBUTs was removed from each chamber and transferred into 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany).

Ultrafiltration (n = 3). Two different UF devices were used, *i.e.* Amicon Ultra 0.5 mL Centrifugal filters and Centrifree Ultrafiltration filters (Merck, Darmstadt, Germany). The membranes of these filters consisted of regenerated cellulose, having a MWCO of 30 kDa. A mixed stock solution with theoretical concentrations of 7 (HA), 0.7 (IAA), 2.6 (IS) and 3.5 (*p*CS) mg/L was prepared in PBS buffer and 260 and 150 µL were transferred into the Amicon and Centrifree filters, respectively. Subsequently, Amicon filters were spun at 4520 *g* during 20 min at RT whereas Centrifree filters were spun at 1885 *g* during 30 min at RT (Beckman Coulter X-15R), as based on Stove *et al.* [4]. Next, ultrafiltrate was transferred into 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany).

Control samples. For each stock solution, a control sample was analyzed without ED or UF to determine the NSA, by calculating the recovery after ED or UF. These stock solutions were prepared accordingly in order to exclude possible differences due to sample preparation.

Finally, 100 µL of each sample (*i.e.* plasma, buffer and ultrafiltrate samples) was treated in the same way. First, the sample was diluted with 260 µL HPLC water, heated at 95 °C (30 min) to precipitate the proteins, cooled down on ice (10 min) and centrifuged (7379g, 10 min) [5].

3.2.3 Results and discussion

The recovery of IAA, IS and *p*CS were in the range of 99.9 – 104.7 % in ED experiments. A lower recovery was found for HA (*i.e.* 88.5 %), but was still acceptable as typically a limit of

± 15 % is accepted in validation guidelines [6,7]. In UF experiments, the recovery of all 4 PBUTs was in the range of 100.9 – 104.1 % for both types of filters (Table 3-1).

Table 3-1 Recovery (%) of 4 protein-bound uremic toxins [hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS)] as determined in a stock solution after equilibrium dialysis (ED) and ultrafiltration (UF), using two different UF filters.

	ED	UF (Amicon)	UF (Centrifree)
HA	88.5 ± 4.7	100.9 ± 3.9	104.1 ± 4.3
IAA	99.9 ± 5.8	103.3 ± 6.4	103.8 ± 2.0
IS	100.8 ± 5.8	102.4 ± 6.3	103.0 ± 2.1
*p*CS	104.7 ± 6.0	101.5 ± 6.1	103.0 ± 1.9

Values are expressed as mean ± standard deviation.

3.2.4 Conclusion

From this experiment, it was concluded that the investigated PBUTs show no NSA to the used ED and UF membranes.

3.3 Comparison of equilibrium dialysis and ultrafiltration to obtain free protein-bound uremic toxin concentrations

3.3.1 Introduction

ED is often regarded as the reference method to obtain free fractions of a protein-bound analyte. A major drawback, however, is the time needed for equilibration, *i.e.* 5 – 6 hours for small compounds or even 24 hours for large compounds. Because of its shorter analysis time, which is typically 20 – 30 min, UF has gained more interest [2,3,8].

Different UF settings, such as centrifugation time and *g*-force, can affect the %PB of an analyte and final settings should be chosen in such a way that obtained free concentrations (and thus %PB) are comparable to those obtained by ED [9].

In this thesis, both ED and UF were used to determine free PBUT concentrations. Therefore, a small experiment was conducted on the comparison of both methods to obtain free PBUT concentrations.

3.3.2 Materials and methods

3.3.2.1 Sample Collection

Pre-dialysis blood samples from hemodialysis patients (n = 6) were collected in Vacutainer K_2EDTA tubes (Becton Dickinson Company, New Jersey, USA). Subsequently, blood was centrifuged for 10 min at 2095*g* at RT (Beckman Coulter X-15R centrifuge - VWR, Leuven, Belgium) and the obtained plasma was stored at -80 °C.

This experiment was conducted according to the Declarations of Helsinki, was approved by the Ethical Committee of Ghent University Hospital (2017/0162) and all participants gave their written informed consent.

3.3.2.2 Chemicals

PBS tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA) and water (HPLC grade) was purchased from Acros Organics (Thermo Scientific, Geel, Belgium).

3.3.2.3 Equilibrium dialysis and ultrafiltration

Equilibrium dialysis (*n* = 6). An HTDialysis 96b system (HTDialysis, Connecticut, USA) system was used. The dialysis membranes, consisting of regenerated cellulose with a MWCO of 12-14 kDa, were used according to the manufacturer's guidelines. Plasma samples (150 µL) were dialyzed against PBS buffer (150 µL, pH = 7.4) for 5 h at 37 °C on a reciprocating shaker. Subsequently, the plasma and PBS samples from each chamber were transferred into 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany). Total PBUT concentrations were measured in native plasma samples, whereas free concentrations were determined by multiplying free fractions and total concentrations. As described in 2.2.2 Sample preparation, these free fractions were calculated from the concentrations measured in the samples at both sides of the ED membrane.

Ultrafiltration (*n* = 6). Amicon Ultra 0.5 mL Centrifugal filters (Merck, Darmstadt, Germany) with a membrane MWCO of 30 kDa were filled with plasma (260 µL) and spun at 4520 *g* during 20 min at 37 °C. Subsequently, ultrafiltrate was transferred into 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany). Total and free concentrations were measured in the native plasma and the ultrafiltrate, respectively.

Finally, 100 µL of each sample (*i.e.* plasma, buffer and ultrafiltrate samples) was treated in the same way. First, the sample was diluted with 260 µL HPLC water, heated at 95 °C (30 min) to precipitate the proteins, cooled down on ice (10 min) and centrifuged (7379g, 10 min) [5].

3.3.2.4 Calculations

The %PB was calculated from the measured free (C_F) and total (C_T) concentration:

$$\%PB = \left(1 - \frac{C_F}{C_T}\right) \times 100\ \% \qquad \text{(Eq. 3-1)}$$

Statistical evaluation was performed with GraphPad Prism 4.00 for Windows (GraphPad Software, La Jolla, California USA). Data were checked for normality by a Shapiro-Wilk test and Wilcoxon signed-rank tests were used to compare the C_F and %PB as obtained by ED and UF. $P < 0.05$ was considered significant.

3.3.3 Results and discussion

Total PBUT concentrations measured in the native plasma samples, free PBUT concentrations determined after separation by ED and UF as well as the calculated %PB are tabulated in Table 3-2. The total PBUT concentrations reflect the large inter-patient variability, especially for HA. For all PBUTs, the free concentrations obtained by ED and UF were comparable, resulting in comparable %PB values between ED and UF.

Table 3-2 Total (C_T) and free (C_F) concentrations as well as the percentage protein binding (%PB) of 4 protein-bound uremic toxins [hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS)] as determined in plasma from 6 hemodialysis patients either immediately (C_T) or after equilibrium dialysis (ED) and ultrafiltration (UF) for C_F.

	C_T (mg/dL)	C_F (mg/dL)		%PB (%)	
		ED	UF	ED	UF
HA	3.23[2.55;5.20]	1.47[1.24;2.57]	1.38[1.07;2.76]	53.3[48.6;51.5]	54.2[50.2;55.5]
IAA	0.16[0.15;0.19]	0.041[0.037;0.044]	0.044[0.040;0.055]	75.6[71.9;78.5]	74.1[72.8;75.5]
IS	1.86[0.96;2.44]	0.13[0.09;0.24]	0.09[0.05;0.17]	90.6[88.8;91.4]	93.7[93.3;94.5]
***p*CS**	3.00[1.91;4.18]	0.26[0.14;0.33]	0.25[0.18;0.36]	92.5[89.2;93.4]	93.4[87.8;94.0]

Values are expressed as median [25th percentile (pct); 75th pct]; For IS n = 5 due to a visual outlier.

3.3.4 Conclusion

From this experiment, it was concluded that both ED and UF, at the tested settings, gave comparable results for both C_F and %PB for HA, IAA, IS and *p*CS.

3.4 Effect of sample temperature, pH and matrix on the percentage protein binding of protein-bound uremic toxins

Based on: Deltombe O, Dhondt A, Van Biesen W, Glorieux G, Eloot S (2017) Effect of sample temperature, pH, and matrix on the percentage protein binding of protein-bound uraemic toxins. Anal Methods 9:1935–1940. doi: 10.1039/C7AY00054E.

3.4.1 Abstract

While studying and trying to optimize dialysis clearances of protein-bound uremic toxins (PBUTs), the percentage protein binding (%PB) might be an important parameter and can be calculated from measured free and total concentrations. Since different parameters may alter this %PB, we investigated whether the ultrafiltration temperature, sample pH and sample matrix (*i.e.* serum or plasma) affect the %PB of PBUTs. Pre-dialysis serum and plasma samples were obtained from 10 stable hemodialysis patients. Ultrafiltration was performed at 37 °C for fresh samples and at 4 °C, room temperature and 37 °C for thawed samples and sample pH was determined (all $n = 10$). Total and free serum/plasma concentrations of hippuric acid, indole-3-acetic acid, indoxyl sulfate and *p*-cresyl sulfate were simultaneously measured by high-performance liquid chromatography with ultraviolet and fluorescence detection. No differences in %PB were found between fresh and thawed samples at 37 °C or between serum and plasma samples prepared at the same temperature despite a difference in pH. However, in both serum and plasma samples, the free concentration increased with increasing ultrafiltration temperatures and resulted in a decrease in %PB from 4 °C to 37 °C. In conclusion, the %PB of PBUTs can be determined in both thawed serum and plasma samples and ultrafiltration should be performed at 37 °C.

3.4.2 Introduction

A myriad of compounds are retained in patients suffering from chronic kidney disease (CKD). Amongst these compounds, some interact negatively with biological functions and are called uremic toxins. Based on their physico-chemical properties, three classes of uremic toxins can be distinguished: (i) the free small water-soluble compounds [molecular weight (MW) < 500 Da], (ii) the middle molecules (MW > 500 Da) and (iii) the protein-bound solutes [10,11]. Many of these protein-bound solutes contribute to the increased inflammation and cardiovascular morbidity and mortality in CKD patients [12–19]. Furthermore, the removal of

these protein-bound uremic toxins (PBUTs) is hampered during dialysis, especially for those uremic toxins with high protein binding, e.g. indoxyl sulfate or *p*-cresyl sulfate [20–22]. Due to the importance of their toxic effects and the hampered removal during dialysis, research on these PBUTs is timely and relevant.

Recently, Arund *et al.* developed a method for the online measurement of fluorophores, like tryptophan and indoxyl sulfate, in spent dialysate during hemodialysis by coupling a spectrofluorometer to the drain outlet of the dialysis machine [23]. Besides this indirect method, no other methods are currently available to analyze uremic toxins and the percentage of protein binding (%PB) *in-vivo*. In order to measure these toxin concentrations, blood sampling is required; serum or plasma tubes are most often centrifuged and aliquots of the supernatant are usually stored at -80 °C until batch analysis. Over the years, several methods have been developed to analyze PBUTs, including gas and liquid chromatography, either using an ultraviolet, fluorescence or mass spectrometer as detector [24–28]. Since no techniques are currently available to directly estimate the %PB, an extra step during sample preparation needs to be introduced. In this step, equilibrium dialysis (ED) or ultrafiltration (UF) is used to separate the free and the protein-bound fractions. The %PB can then be calculated from the measured free and total concentrations.

The binding of solutes to proteins is considered to be a dynamic equilibrium between the free and bound fractions, and is reported to vary from 10 % (e.g. *p*-cresyl glucuronide) to nearly 100 % (e.g. *p*-cresyl sulfate) for PBUTs. This equilibrium is known to be influenced by the pH of the serum or plasma samples and has been studied for a large number of drugs [3,9,29,30]. An increase in pH is caused by diffusion of CO_2 out of the serum or plasma and may occur when these samples are unsealed or undergo one or multiple freeze/thaw cycles [31].

The percentage of protein binding is also influenced by the temperature. Consequently, temperature control might be important during the processing of samples by UF (duration 20-30 min) or even more in ED (duration 4-6 h) to avoid bias. In previous studies, several temperature conditions during ED or UF have been compared for various drug compounds, and 37 °C is typically selected as a standard since it best reflects the physiological condition [3,4,9,32].

Parameters that may alter the %PB of PBUTs should be known when studying these solutes. However, to the best of our knowledge, only a few studies to date investigated whether pH and temperature – two parameters known to alter the %PB for various drug compounds – have an

influence on the %PB of PBUTs [33–35], and whether these findings can also be extrapolated to uremic patients, as it can be postulated that there is a different interaction with uremic proteins. Therefore, in this study, we compared the following in samples of uremic patients: (i) the impact of pH on %PB in fresh and thawed serum and plasma samples; (ii) the %PB when UF was performed at 37 °C, room temperature (RT) and 4 °C; and (iii) the pH and %PB between serum and plasma samples, prepared at the same temperatures.

3.4.3 Materials and methods

3.4.3.1 Sample collection and preparation

Blood samples were collected from 10 stable hemodialysis patients in Venosafe serum (Terumo Europe, Leuven, Belgium) or Vacutainer K_2EDTA (Becton Dickinson Company, New Jersey, USA) tubes. Serum tubes were allowed to clot, whereas plasma tubes were put on ice immediately after collection. These tubes were centrifuged (2095*g*, 10 min) and aliquots of 500 µL were immediately used or frozen at -80 °C until further sample preparation. This study was performed according to the Declarations of Helsinki, approved by the Ethical Committee of Ghent University Hospital (EC 2015/0932) and all patients gave their written informed consent.

The effect of pH, temperature and sample matrix (*i.e.* serum or plasma) on the %PB of PBUTs was investigated using the following protocol. Immediately after collection (fresh 37 °C) or thawing (37 °C, RT or 4 °C) at 4 °C, serum (n = 10)/plasma (n = 10) samples and Amicon Ultra 0.5 mL Filters (molecular weight cut-off 30 kDa, Merck KGaA, Darmstadt, Germany) were both adjusted to the right temperature for 30 min at either 37 °C, RT or 4 °C, followed by pH measurement of each sample with a WTW pH 330 instrument (Weilheim, Germany). Subsequently, 260 µL of sample was filtered (4520 *g*, 20 min) through the Amicon filters at either 37 °C, RT or 4 °C. The ultrafiltrate (100 µL) containing the free fraction was further diluted with 260 µL HPLC water and heated at 95 °C during 30 min, cooled down on ice (10 min) and centrifuged (15588 *g*, 10 min). In this way, all steps were equal to those for total toxin concentration determination.

Untreated serum/plasma samples (100 µL) were first diluted with 260 µL HPLC water, followed by a protein denaturation step (95 °C, 30 min) to determine the total concentration.

After heating, the samples were cooled down on ice for 10 min. Subsequently, the samples were centrifuged (15588 *g*, 10 min) and filtered (4520 *g*, 20 min) through the Amicon filters.

Finally, each sample (225 µL) was transferred into an autosampler vial and frozen at -80 °C until batch analysis. Prior to analysis, the samples were thawed, 25 µL of internal standard (fluorescein, 50 mg/L) was added in order to correct for instrument performance variations and the mixture was vortexed. All samples were kept at 4 °C in the autosampler and 18 µL was injected onto the column.

3.4.3.2 Chemicals

Hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and fluorescein were purchased from Sigma-Aldrich (St. Louis, MO, USA) and *p*-cresyl sulfate (*p*CS) from TCI Chemicals (Zwijndrecht, Belgium). Water and methanol, both HPLC grade, were purchased from Acros Organics (Thermo Scientific, Geel, Belgium), formic acid from VWR (Leuven, Belgium), and ammonia from Merck (Merck KGaA, Darmstadt, Germany). The chemical structures of HA, IAA, IS and *p*CS are presented in Figure 3-1.

3.4.3.3 Calibration standards

Stock solutions (100 mg/dL) of all compounds were prepared in HPLC water and kept at -80 °C. Seven calibration standards were prepared by spiking 100 µL blank serum with 100 µL stock solution and 160 µL HPLC water, followed by the sample preparation for total concentration. Blank serum was obtained as described by de Loor *et al.* [28].

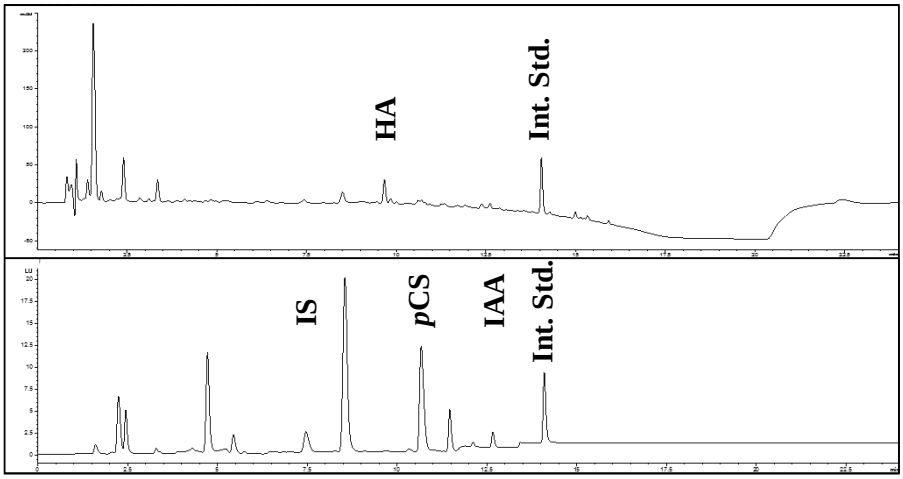
3.4.3.4 Instrumentation

Concentrations of PBUTs were determined on an ultra-high performance liquid chromatography instrument with ultraviolet and fluorescence detection (UPLC-UV/FLD). An Agilent 1290 Infinity device was used and chromatographic separation was performed at 26 °C on a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 x 100 mm) with a Waters Acquity UPLC BEH C18 Van Guard column (1.7 µm, 2.1 x 5 mm). A linear gradient elution was used to separate the compounds at a flow rate of 0.3 mL/min, and started at 98 % ammonium formate buffer (50 mM, pH 3, mobile phase A), followed by a composition change of 90 % A in 7 min. In the next 9 minutes, the mobile phase changed to 100 % methanol (mobile phase B) and was

held for 3 min. Finally, a re-equilibration step was performed. HA was detected with an Agilent G4212A diode array detector at 245 nm. IS (λ_{ex}: 280 nm, λ_{em}: 376 nm), *p*CS (λ_{ex}: 264 nm, λ_{em}: 290 nm), IAA (λ_{ex}: 280 nm, λ_{em}: 350 nm) and fluorescein (λ_{ex}: 443 nm, λ_{em}: 512 nm) were detected by an Agilent G1316C fluorescence detector. A representative chromatogram for total concentration of HA (top), IAA, IS, and *p*CS (bottom) in a serum sample prepared at 37 °C is depicted in Figure 3-2.

The within-run and between-run precisions were determined by using an EDTA uremic plasma pool. From this pool, 6 samples were individually prepared and analyzed in a single run (*i.e.* within-run, n = 6) as well as in different runs performed on 3 different days (*i.e.* between run, n = 6). Subsequently, the relative standard deviation on the measured concentration was calculated, resulting in a within-run and between-run precision of 1.99 % and 3.24 % for HA, 2.56 % and 6.34 % for IAA, 2.58 % and 4.84 % for IS, and 2.52 % and 5.95 % for *p*CS respectively.

Figure 3-2 Representative chromatogram for total concentration of hippuric acid (HA, top, UV detection) and indoxyl sulfate (IS), *p*-cresyl sulfate (*p*CS) and indole-3-acetic acid (IAA) (bottom, fluorescence detection) in a serum sample prepared at 37 °C.



Int. Std.: internal standard.

3.4.3.5 Calculations

The %PB was calculated from the measured free (C_F) and total (C_T) concentration:

$$\%PB = \left(1 - \frac{C_F}{C_T}\right) \times 100\ \% \qquad \text{(Eq. 3-1)}$$

Statistical evaluation was performed with GraphPad Prism 4.00 for Windows (GraphPad Software, La Jolla, California USA). Data were checked for normality by a

Kolmogorov Smirnov test and paired t-tests were used to compare the concentrations (both total and free), pH and %PB between each group. $P < 0.05$ was considered significant.

3.4.4 Results

The total and free concentrations of the measured PBUTs are presented in Table 3-3 and Table 3-4, respectively. Serum and plasma samples prepared at different temperatures, as well as serum and plasma samples prepared at the same temperature, were compared. For IS, a small decrease in total concentration was observed when prepared at 4 °C, compared to the total concentrations when prepared at RT and 37 °C. No differences in total PBUT concentrations were found between the other temperatures and between the different sample matrices at the same temperature. The free concentrations of HA, IAA and IS slightly decreased from 37 °C to RT and were found to be the lowest at 4 °C.

Table 3-3 Total concentration (mg/dL) of four protein-bound uremic toxins in serum and plasma: hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS); freshly prepared (fresh 37 °C) or after 1 freeze/thaw cycle (37 °C, RT and 4 °C).

C_T (mg/dL)		Fresh 37 °C	37 °C	RT	4 °C
HA	*Serum*	1.76 ± 1.48	1.71 ± 1.54	1.67 ± 1.45	1.74 ± 1.57
	Plasma	1.72 ± 1.51	1.68 ± 1.38	1.65 ± 1.42	1.64 ± 1.45
IAA	*Serum*	0.25 ± 0.23	0.23 ± 0.24	0.23 ± 0.22	0.21 ± 0.21
	Plasma	0.23 ± 0.23	0.23 ± 0.21	0.21 ± 0.20	0.21 ± 0.20
IS	*Serum*	1.22 ± 0.51	1.14 ± 0.46	1.12 ± 0.44	0.96 ± 0.41[a,b,c]
	Plasma	1.13 ± 0.45	1.13 ± 0.48	1.07 ± 0.45	0.92 ± 0.38[a,b,c]
*p*CS	*Serum*	3.44 ± 1.85	3.12 ± 1.36	3.12 ± 1.48	2.92 ± 1.30[a]
	Plasma	3.25 ± 1.64	3.23 ± 1.71	3.02 ± 1.39	2.91 ± 1.37

[a] $p < 0.05$ *versus* fresh 37 °C. [b] $p < 0.05$ *versus* 37 °C. [c] $p < 0.05$ *versus* RT.
Values are expressed as mean ± standard deviation.

Table 3-4 Free concentration (mg/dL) of four protein-bound uremic toxins in serum and plasma: hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS); freshly prepared (fresh 37 °C) or after 1 freeze/thaw cycle (37 °C, RT and 4 °C).

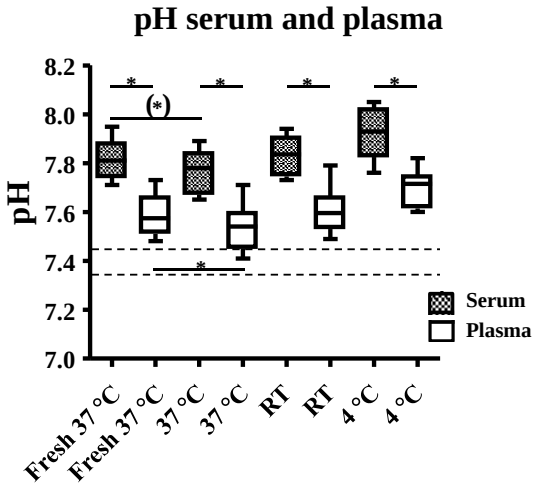
C_F (mg/dL)		Fresh 37 °C	37 °C	RT	4 °C
HA	*Serum*	1.15 ± 1.02	1.07 ± 1.01	1.00 ± 0.93[a,b]	0.89 ± 0.86[a,b,c]
	Plasma	1.04 ± 1.00	1.01 ± 0.97	1.04 ± 1.06	0.90 ± 0.94[a,b,c]
IAA	*Serum*	0.08 ± 0.09	0.08 ± 0.09	0.07 ± 0.08[a]	0.06 ± 0.06[a]
	Plasma	0.08 ± 0.09	0.08 ± 0.08	0.08 ± 0.08	0.05 ± 0.06[a,b,c]
IS	*Serum*	0.13 ± 0.09	0.13 ± 0.09	0.09 ± 0.07[a,b]	0.05 ± 0.04[a,b,c]
	Plasma	0.11 ± 0.07	0.11 ± 0.07	0.08 ± 0.06[a,b]	0.03 ± 0.03[a,b,c]
*p*CS	*Serum*	0.31 ± 0.20	0.36 ± 0.25	0.25 ± 0.14	0.24 ± 0.22[a]
	Plasma	0.28 ± 0.17	0.26 ± 0.14	0.26 ± 0.16	0.20 ± 0.18[b]

[a] $p < 0.05$ *versus* fresh 37 °C. [b] $p < 0.05$ *versus* 37 °C. [c] $p < 0.05$ *versus* RT.
Values are expressed as mean ± standard deviation.

The effect of sample matrix and preparation temperature on the sample pH is illustrated in Figure 3-3. The pH of the plasma samples remained systematically closer to the physiologic

pH (7.35-7.45) compared to the pH of the serum samples. The lowest and most physiologic pH was observed in the plasma samples prepared at 37 °C. A small decrease in pH was found in thawed samples (37 °C) as compared to fresh samples (fresh 37 °C) in both serum (borderline significant with $p = 0.059$) and plasma ($p < 0.001$) samples.

Figure 3-3 Measured pH of serum and plasma samples, prepared fresh (fresh 37 °C) or after 1 freeze/thaw cycle (37 °C, RT, and 4 °C).



* $p < 0.001$, (*) $p = 0.059$.

Table 3-5 summarizes the observed %PB of the four PBUTs in serum and plasma samples, when prepared freshly (fresh 37 °C) or after one freeze/thaw cycle (37 °C, RT and 4 °C). The increase in %PB with decreasing sample preparation temperature was more pronounced in the serum samples, especially for HA, IS, and *p*CS, while in the plasma samples this increase in %PB was only significant for HA and IS at 4 °C.

Table 3-5 Percentage protein binding (%PB) of four protein-bound uremic toxins in serum and plasma: hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS); freshly prepared (fresh 37 °C) or after 1 freeze/thaw cycle (37 °C, RT and 4 °C).

%PB		Fresh 37 °C	37 °C	RT	4 °C
HA	*Serum*	39.4 ± 9.7	42.2 ± 7.2	45.3 ± 6.2[a,b]	49.4 ± 13.8[a,b,c]
	Plasma	45.5 ± 7.2	46.3 ± 11.2	45.3 ± 10.5	53.5 ± 9.0[a,b,c]
IAA	*Serum*	63.7 ± 13.5	63.5 ± 11.6	66.3 ± 10.1[b]	70.1 ± 11.1
	Plasma	64.6 ± 11.9	64.9 ± 13.0	64.2 ± 14.1	72.9 ± 12.3
IS	*Serum*	88.7 ± 6.3	88.0 ± 6.4	91.2 ± 4.7[a,b]	95.0 ± 3.0[a,b,c]
	Plasma	89.9 ± 5.5	89.7 ± 6.0	92.0 ± 4.5[a,b]	96.0 ± 2.7[a,b,c]
*p*CS	*Serum*	90.5 ± 4.1	89.2 ± 4.2	91.6 ± 3.4[a,b]	92.3 ± 4.3[b]
	Plasma	91.2 ± 3.4	91.3 ± 3.7	91.4 ± 3.8	93.6 ± 3.7

[a] $p < 0.05$ *versus* fresh 37 °C. [b] $p < 0.05$ *versus* 37 °C. [c] $p < 0.05$ *versus* RT.
Values are expressed as mean ± standard deviation.

No significant differences in %PB were found in serum or plasma samples when prepared fresh (fresh 37 °C) or thawed (37 °C); similarly, the %PB between serum and plasma samples prepared at the same temperature did not differ significantly (Table 3-5).

3.4.5 Discussion

Establishing the correct %PB of PBUTs is important: (i) to be able to estimate the biological activity of uremic toxins, since the free fraction is considered to be the biologically active one and (ii) in view of their removal during dialysis, because the removal is hampered by the protein binding. Therefore, it is interesting to know to what extent parameters known to influence the %PB of various drug compounds also affect the %PB of PBUTs during sample preparation. To the best of our knowledge, the present study is the first to present the effect of pH, temperature and matrix on the determination of total and free concentrations of PBUTs including HA, IAA, IS and *p*CS. In literature, the use of a variety of sample preparation temperatures (*i.e.* 4 °C, 25 °C, RT and 37 °C) can be found [20,26,35]. Furthermore, the matrix in which the protein-bound uremic toxins are determined differs from the method used [24–28]. Therefore, this work focused on parameters that, in the field of protein-bound drugs, are known to have an influence on the %PB [3,4,9,29,30,32,36].

The present study reveals that total concentrations in serum and plasma did not differ, except for IS at 4 °C, where a significantly lower concentration was observed. Although we have no explanation for this observation, we suggest that this is only specific for IS at 4 °C, since the total concentration did not decrease for HA, IAA and *p*CS at 4 °C.

The small, but significant difference in pH between the freshly prepared (fresh 37 °C) and thawed samples at 37 °C did not affect the %PB. Consequently, serum or plasma samples can be frozen and thawed for at least once, without a change in %PB. This is an important observation, since most research groups store their samples at -80 °C to perform batch analysis to avoid the introduction of bias caused by differences between batches.

Notwithstanding the larger difference in pH between serum and plasma samples, no differences in %PB were observed. This implicates that both serum and plasma samples can be used to determine the %PB for these compounds. Sakai *et al.* reported that even larger differences in pH (*i.e.* 6.5, 7.4 and 8.5 at 25 °C) have only a slight effect on the binding affinity constant (and thus the %PB) for IS to human serum albumin (HSA) in solution [34]. For IAA, Bertuzzi *et al.* found that the affinity constant to HSA changes in extreme pH conditions (5.0, 7.4 and 8.5)

[33]. Except for pH 7.4, the pH conditions described by Sakai *et al.* and Bertuzzi *et al.* are physiologically irrelevant and are too extreme. In the present work, pH 8.5 was never reached because at least one factor that affects the pH, *i.e.* exposure to the air, was minimized by keeping the tubes sealed. Additionally, it was shown that the %PB was not influenced in the range of pH 7.4 – 8.0.

The free concentration increased with increasing ultrafiltration temperatures (Table 3-4), resulting in a lower %PB at 37 °C. This is in accordance with Viaene *et al.* for IS and *p*CS [35], although the %PB of *p*CS was not found to be significantly decreased in our work. For IAA, no decrease in %PB was found with increasing temperatures. However, 37 °C should be the temperature of choice when analyzing PBUTs in general because this best reflects the physiological condition. Nevertheless, the difference in %PB at RT and 37 °C was rather small, and performance of UF at RT when no temperature-controlled centrifuge is available might be justifiable.

The chromatographic method used in this study does not allow to correct for variations during the sample preparation step. The internal standard used (fluorescein) was added just before the chromatographic analysis, because the molecule binds to proteins and adsorbs to the Amicon filters (data not shown). Ideally, each analyte should have an internal standard to correct for and the standard should be added at the start of the sample preparation. If the lower total IS concentration at 4 °C was caused by loss of IS during the sample preparation, this could possibly be corrected by addition of an internal standard at the start. To date, no alternative internal standard for the current method is available. However, both the total concentration range and %PB measured by our method are in line with values found in literature, in which deuterated internal standards for HA, IAA, IS and *pCS* were added at the start of the sample preparation and samples were analyzed with mass spectrometry [26,28].

3.4.6 Conclusion

In this study, we investigated whether the pH, temperature, or the type of sample matrix affects the determination of total and free concentrations, and thus the %PB, of four PBUTs including HA IAA, IS and *p*CS. Our findings revealed that the %PB of PBUTs can be determined in both thawed serum and plasma samples, but UF should be performed at 37 °C.

3.4.7 Acknowledgements

This study was supported by The Research Foundation – Flanders (FWO Vlaanderen, Project Number G0A4614N) and the authors acknowledge Sophie Lobbestael for her laboratory assistance.

3.5 Development and validation of an UHPLC – high resolution MS method for the quantification of total and free teicoplanin in human plasma

In preparation: Deltombe O, Mertens T, Eloot S and Verstraete A G, Development and validation of an UHPLC – high resolution MS method for the quantification of total and free teicoplanin in human plasma.

3.5.1 Abstract

The antibiotic teicoplanin, used for the treatment of infections caused by Gram-positive bacteria, is highly bound to plasma proteins (percentage protein binding, %PB, around 90 %) and therapeutic plasma levels of total teicoplanin are 10 – 100 mg/L. Because of the low free concentrations (*i.e.* < 1 – 10 mg/L), current immunoassays are not able to quantify free teicoplanin concentrations, although it might be more relevant in therapeutic drug monitoring than total concentrations.

In this study, an ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS) method for the quantification of total and free teicoplanin in K_2EDTA plasma samples was developed and validated. Furthermore, %PB obtained by ultrafiltration was compared with that obtained by equilibrium dialysis using spiked samples from healthy subjects.

Analytes were separated using a phenylhexyl column, gradient mobile phase analysis was used, total run time was 4.5 min and teicoplanin was detected by orbitrap MS. The precision and accuracy were below 15 % and within ± 15 %, respectively and teicoplanin was found to be stable for at least 14 days in plasma at 4 °C. The %PB of teicoplanin in spiked plasma from healthy subjects as obtained by ultrafiltration (94.1 ± 1.3 %) was in good agreement with that obtained by equilibrium dialysis (93.6 ± 0.4 %), whereas mean %PB of teicoplanin in samples from infected patient who received the antibiotic was 87.7 ± 4.2 % (range: 79.6 – 95.4 %).

A novel highly sensitive UHPLC-HRMS method was developed and validated for the quantification of total and free teicoplanin in human K_2EDTA plasma samples. Amongst others, this method is suitable for therapeutic drug monitoring.

3.5.2 Introduction

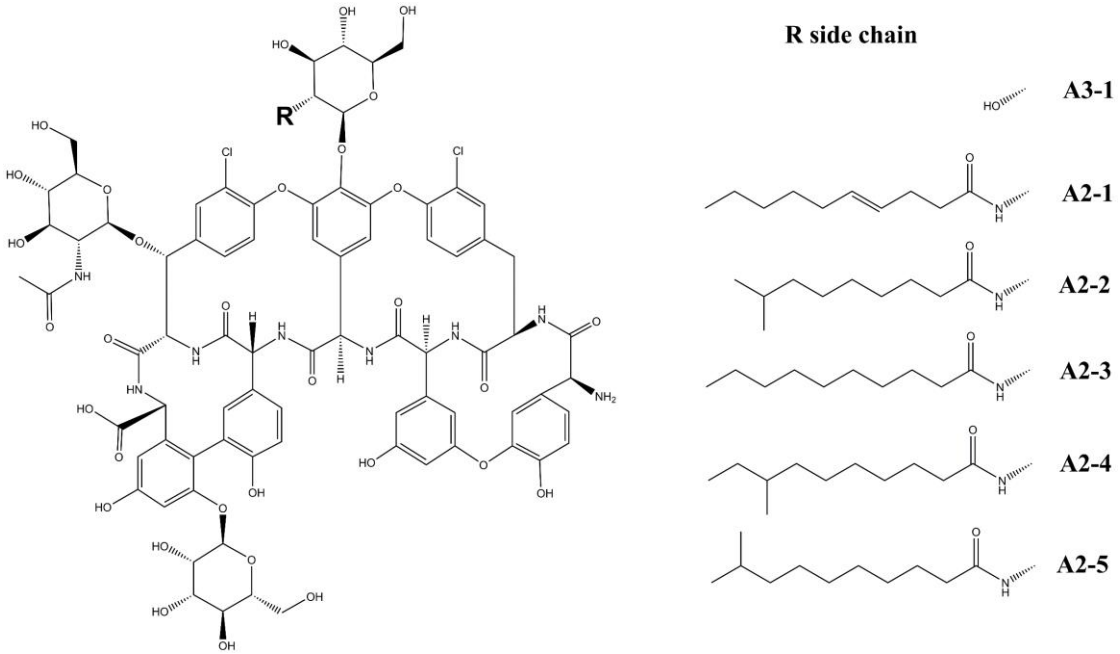
It is considered that only the free drug fraction is biologically active because only this fraction can travel through cell membranes, by e.g. passive diffusion, towards the site of action [37]. Alterations in free fractions can be of clinical relevance, especially for drug compounds with a high level of protein binding having a narrow therapeutic range or when drug resistance is possible. A change in percentage protein binding (%PB) from e.g. 95 % to 90 % implicates a doubling in free fraction and may have an impact on the pharmacokinetics (PK) and pharmacodynamics (PD) of that drug. Therefore, knowledge of the free drug concentrations may be more relevant than total concentrations to fully understand PK/PD as for instance with therapeutic drug monitoring (TDM) [38–40].

The most widely used methods to separate free fractions are equilibrium dialysis (ED) and ultrafiltration (UF) [2, 3, 8]. Although ED is considered to be the reference, its major drawback is the long equilibration time (*i.e.* equilibration of the free fraction between the serum/plasma and buffer chamber), which is 5 – 6 hours for small compounds or even 24 hours for large compounds. In addition, equilibration time also depends on the molecular weight cut-off of the membrane. Conversely, UF is a more rapid method and separates the free fraction in only 20 – 30 min by centrifugation, which forces water and free drugs through the filter membrane. It should however be mentioned that both ED and UF sometimes suffer from non-specific adsorption (NSA) to the used membrane. In ED, this can be solved by measuring the concentration in both the buffer and serum/plasma chamber, whereas in UF, correction for NSA can be readily performed by filtering the calibration standards, together with the unknown samples.

Besides vancomycin, the glycopeptide antibiotic teicoplanin can also be used for the treatment of infections caused by Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) [41–44]. Teicoplanin is composed of a mixture of compounds which only differ in the alkyl side chain (see Figure 3-4), including 5 major (A2-1 to 5) compounds accounting for 90 – 95 % of the total product, a more polar compound (A3-1) and 4 minor (RS-1 to 4) compounds [41, 45] binds to plasma proteins, with a %PB reported to range from around 70 to 99 % [38, 46, 47] and is less toxic than vancomycin, particularly in terms of nephrotoxicity [48,49]. Therapeutic levels of total teicoplanin in plasma range from 10 to 100 mg/L [50–52] and hence, theoretical free teicoplanin concentrations range from below 1 up to 10 mg/L [38, 46]. Due to the small number of studies, data correlating total teicoplanin

concentrations to clinical outcome are limited and even absent for free teicoplanin [53,54]. Nevertheless, TDM is important to ensure its therapeutic effectiveness [47,52]. However, since the free drug fraction is responsible for therapeutic effects and the inter-patient variability is large [38, 39], free concentration monitoring might be more relevant than total concentration. Despite this theoretical importance of monitoring free teicoplanin concentrations, no clinical studies have confirmed this so far.

Figure 3-4 Chemical structures of the 5 different major teicoplanin compounds, A2-1 to A2-5, and the more polar compound A3-1, which only differ in the alkyl side chain on the core group.



Throughout the years, different analytical methods have been developed for the quantification of total teicoplanin in serum or plasma samples, including fluorescence polarization (FPIA) [46,55–57] and homogeneous particle-enhanced turbidimetric [58] immunoassays as well as high performance liquid chromatography techniques in which teicoplanin was quantified by ultraviolet detection [38] or mass spectrometry [59–61]. In the small number of papers discussing the quantification of free teicoplanin, there was no evidence for the accuracy of the obtained free fractions (e.g. by comparison with ED) or these free fractions were obtained at physiologically irrelevant temperatures (4 °C) [38,46,56].

Therefore, we developed and validated a sensitive method for the quantification of total and free teicoplanin in plasma samples by ultra-high performance liquid chromatography – high

resolution mass spectrometry (UHPLC-HRMS). In our method, free fractions were obtained by UF at 37 °C and the used UF settings were compared with the reference method ED.

3.5.3 Materials and methods

3.5.3.1 Chemicals

Acetone, acetonitrile, methanol and formic acid (all LC-MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Teicoplanin European pharmacopoeia reference standard (CRS 50), ammonium formate and trimipramine-D_3 100 mg/L in methanol were obtained from Sigma Aldrich (Saint-Louis, MO, USA). The teicoplanin reference standard consisted of 5 major compounds (with different relative abundances), including teicoplanin A2-1 (6.2%), isomers A2-2 & A2-3 (67.5%) and isomers A2-4 & A2-5 (21.1%) as well as the more polar compound A3-1 (5.2%) (Figure 3-4). Ultrapure water (conductivity < 1 µS and filtered through a 45 µm filter) was used for all dilutions and prepared using an Elga Medica R7 instrument (Veolia water solutions & Technologies, Ede, The Netherlands).

3.5.3.2 Sample Collection

Blood samples from healthy subjects (n = 6) and infected patients (n = 41) to whom teicoplanin was administered were collected in K_2EDTA and lithium heparin plasma tubes as well as in serum separating tubes (SST) (all Becton Dickinson Company, New Jersey, USA). Subsequently, blood was centrifuged for 10 min at 2095 *g* at room temperature (RT) (Beckman Coulter X-15R centrifuge - VWR, Leuven, Belgium) and the obtained plasma and serum was stored at -80 °C. The method described here was developed and validated using K_2EDTA plasma only. Lithium heparin plasma and serum were only used to study the effect of blood tube type on the quantification of teicoplanin, for method comparison and to determine the %PB of teicoplanin in patient samples.

This study was conducted according to the Declarations of Helsinki, was approved by the Ethics Committee of Ghent University Hospital (2017/0162 for healthy volunteers and 2015/004 for patients) and all participants gave their written informed consent.

3.5.3.3 Teicoplanin standards

A stock solution of teicoplanin European pharmacopoeia reference standard with a theoretical concentration of 1400 mg/L was prepared and used for both total and free teicoplanin analyses. A pool of K_2EDTA plasma from healthy volunteers (*i.e.* blank plasma) was used to prepare calibration curves.

For the determination of total teicoplanin, 8 calibrator stock solutions (10x the final concentration) were prepared and for each calibrator level, 100 µL was added to 900 µL blank plasma. For free teicoplanin, 7 calibrator stock solutions (10x solution of the final concentration) were prepared and 100 µL was added to 900 µL ultrafiltrate, which was obtained by ultrafiltration of blank plasma using Centrifree Ultrafiltration filters (Merck, Darmstadt, Germany; 30 min, 1885 *g*, RT – Beckman Coulter X-15R centrifuge). Subsequently, these calibrator levels in ultrafiltrate were filtered under the same conditions in order to account for NSA to the UF membrane (see further).

For each calibrator level, aliquots of 50 µL were transferred into 1.5 mL Eppendorf tubes (Hamburg, Germany) and stored at -80 °C until analysis.

3.5.3.4 Sample preparation

The internal standard trimipramine-D_3 (50 µL, 0.5 mg/L in acetone) was added to either 50 µL calibrator prepared in blank plasma (for calibration) or unknown plasma samples for total teicoplanin analysis or to 50 µL calibrator prepared in blank plasma ultrafiltrate (for calibration) or ultrafiltrate samples (obtained by Centrifree filters, 30 min, 1885 *g*, 37 °C – Beckman Coulter X-15R centrifuge) for free teicoplanin analysis, followed by thorough mixing. Subsequently, 200 µL of a cold (4 °C) solution containing methanol and acetonitrile (50/50 v/v %) was added to precipitate the proteins and the samples were mixed again. After cooling down (30 min, -20 °C), each sample was mixed, placed in a Thermomixer for 5 min at 1400 rpm and at 10 °C (Eppendorf, Hamburg, Germany) and centrifuged (5 min, 16162 *g*, RT – Beckman Coulter Microfuge 16). Finally, 100 µL supernatant was transferred into an autosampler vial and placed in the autosampler at 10 °C.

3.5.3.5 UHPLC-HRMS analysis

The chromatographic system consisted of a Dionex Thermo Scientific UltiMate 3000 system (Waltham, Massachusetts, USA) equipped with a binary pump and autosampler with thermostat, which was set at 10 °C. The injection volume was 1 µL and 2 µL for total and free teicoplanin, respectively. Chromatographic separation was performed at 40 °C using an Accucore phenylhexyl column (100 x 2.1 mm, 2.6 µm particle size) (Thermo Scientific, Waltham, Massachusetts, USA). A linear gradient elution was used at 0.4 mL/min and started at 75% of mobile phase A (2 mM ammonium formate and 0.1% formic acid in water) and 25% of mobile phase B (2 mM ammonium formate, 0.1% formic acid, and 1% water in 50/50 v/v% methanol/acetonitrile). Subsequently, the composition of the mobile phase was changed to 80% B during the first 2.5 minutes and was maintained for one minute. Finally, a composition change to 90 % B was achieved in 0.25 min and maintained for 0.75 min, followed by re-equilibration of the column at 75 % A during 0.5 min. The total run time was 4.5 min.

Analytes were detected using a Q-Exactive Hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). Heated electrospray ionization was used in positive mode at a sheath gas (N_2) flow of 45 (arbitrary units, a.u.), a temperature of 300 °C and a spray voltage of 3.5 kV. Auxiliary gas (N_2) flow and temperature were 15 a.u. and 350 °C, respectively. Full scan analysis (m/z 200 – 2000) was applied for total teicoplanin whereas three scan segments were introduced (Table 3-6) for free teicoplanin to increase sensitivity. Automatic gain control (AGC) targets were 1 10^6 and 2 10^5 for total and free teicoplanin, respectively. For both teicoplanin analyses, the resolution was set at 70000. Possible shifts in the mass spectrum were corrected by lock mass at m/z 391.284. Teicoplanin compounds A2-2 & A2-3 and teicoplanin compounds A2-4 & A2-5 are both pairs of isomers and were therefore detected simultaneously, whereas compounds A2-1 and A3-1 were detected separately, as summarized in Table 3-6. Teicoplanin compounds A2-1 to 5 and A3-1 were summed up for calibration as well as for unknowns. Minor compounds (RS1 – 4) were not included. Data acquisition and processing were performed using TraceFinder (Thermo Scientific, Waltham, Massachusetts, USA) software, version 3.3.

Table 3-6 Retention times (t_R) and instrument settings. For each teicoplanin compound, z = 2.

	t_R (min)	m/z 1	m/z 2	m/z 3	m/z 4	m/z 5
Scan segment 1: 0.0 – 1.4 min (m/z 780 – 788)						
A3-1	0.83	782.183	782.685	783.183	783.683	784.184
Scan segment 1: 1.4 – 2.1 min (m/z 936 – 951)						
A2-1	1.72	938.778	939.280	939.779	940.280	940.780
A2-2 & A2-3	1.72	939.786	940.287	940.786	941.286	941.786
A2-4 & A2-5	1.87	946.793	947.295	947.794	948.295	948.794
Scan segment 1: 2.1 – 4.5 min (m/z 290 – 300)						
Internal standard	2.35	298.236	n.a.	n.a.	n.a.	n.a.

m/z: mass-to-charge ratio; total teicoplanin: only underlined m/z values;
free teicoplanin: scan segments and m/z 1 to 5;
n.a.: not applicable.

3.5.3.6 Equilibrium dialysis versus ultrafiltration

Free fractions of spiked healthy plasma were obtained by both ED and UF. For ED, an HTDialysis 96b system (HTDialysis, Connecticut, USA) was used with membranes of regenerated cellulose (molecular weight cut-off of 12-14 kDa). These membranes were hydrated and used according to the manufacturer's guidelines. Dialysis was performed against blank plasma ultrafiltrate during 24 h at 37 °C (equilibration time was determined in a pilot experiment, data not shown). An adhesive film was used to seal the ED wells to avoid water losses through evaporation and shifts in pH.

Two different UF devices were checked for NSA, *i.e.* Amicon Ultra – 0.5 mL Centrifugal filters and Centrifree Ultrafiltration filters (both Merck, Darmstadt, Germany). Amicon filters were spun at 4520 *g* during 20 min at RT (based on [5]) whereas Centrifree filters were spun at 1885 *g* during 30 min at RT or 37 °C (Beckman Coulter X-15R), as previously described to obtain free vancomycin [4].

Non-specific adsorption of teicoplanin to both the ED device and membrane and to both UF membranes was checked by determining the recovery after ED or UF at RT, using calibrator level 6 for free teicoplanin quantification (*i.e.* 14 mg/L) which was prepared in blank plasma ultrafiltrate (all $n = 3$).

Free (C_F) teicoplanin concentrations in spiked plasma samples obtained by ED (37 °C) and UF (RT and 37 °C, Centrifree filters only) were compared. Additionally, the %PB was calculated from the measured total (C_T) and C_F concentrations: $\%PB = [1 - (C_F/C_T)] \times 100\%$. For this experiment, blank plasma samples from 6 healthy volunteers were individually spiked with teicoplanin to reach final concentrations of either 7 (low), 70 (medium) or 140 (high) mg/L, with each level in duplicate.

3.5.3.7 %PB in patient samples

%PB of teicoplanin was also determined in anonymized left-over plasma (K_2EDTA and lithium heparin) and serum samples (n = 41) from patients who received teicoplanin, which were collected in the core laboratory of Ghent University Hospital.

3.5.3.8 Quality control samples

In house quality control (QC) samples (low, medium and high concentration) were prepared in a pool of blank plasma (total teicoplanin) or in blank plasma ultrafiltrate (free teicoplanin). For total teicoplanin, theoretical concentrations were 7 mg/L (QCL_T), 70 mg/L (QCM_T) and 140 mg/L (QCH_T) whereas for free teicoplanin, theoretical concentrations were 0.93 mg/L (QCL_F), 5.6 mg/L (QCM_F) and 12.6 mg/L (QCH_F).

3.5.3.9 Assay validation

Both total and free teicoplanin assays were validated according to the European Medicines Agency (EMA) guidelines for selectivity, carry-over, limit of quantification (LOQ), calibration curves, accuracy (within-run and between-run), precision (within-run and between-run), matrix effect, storage and freeze/thaw stability [6].

Selectivity for both teicoplanin and internal standard was demonstrated by using 6 individual blank K_2EDTA plasma samples and QC samples, respectively. For both selectivity validations, 50 µL internal standard was replaced by 50 µL ultrapure water. In addition, selectivity was tested in patient samples collected in different blood tubes types, *i.e.* K_2EDTA, lithium heparin and serum separating tubes (SST). Absence of interfering compounds was accepted when the response was < 20 % of LOQ for teicoplanin and < 5 % for the internal standard.

For carry-over effects, six individual blank plasma samples were injected after the highest calibrator. Carry-over was assumed to be negligible when the response was < 20 % of LOQ for teicoplanin and < 5 % for the internal standard.

The LOQ was considered to be the lowest calibration standard and was run 3 times. The LOQ was accepted if the response was at least 5 times the signal of a blank sample.

Calibration curves were evaluated when prepared in blank plasma and blank plasma ultrafiltrate for total (eight levels) and free (seven levels) teicoplanin, respectively (n = 5). A separate

calibration curve was constructed for each teicoplanin compound (*i.e.* teicoplanin A3-1, A2-1, A2-2 & A2-3 and A2-4 & A2-5) and data shown here are the sum of these compounds. Results were expressed as coefficients of variation (CV%) and the bias (%) to the theoretical value was calculated. The calibration curves were accepted when the back calculated concentrations of the calibrator levels were within ± 15 % of the theoretical value, except for the LOQ for which ± 20 % was accepted. The reproducibility was accepted when ≤ 15 % for all levels and ≤ 20 % for the LOQ.

Precision and accuracy were assessed using *in house* made QC samples for both total and free teicoplanin. For within-run precision and accuracy, $LOQ_{T\&F}$, $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ were prepared and analyzed in one batch. Between-run precision and accuracy were determined by preparing $LOQ_{T\&F}$, $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ after 1, 4, 7 and 14 days storage at -80 °C. Also here, results were expressed as CV% and the bias (%) to the theoretical value was calculated. The assays were assumed to be precise and accurate when the CV% and bias were ≤ 15 % for $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ and ≤ 20% for $LOQ_{T\&F}$ and within ± 15 % for $QCL_{T\&F}$, $QCM_{T\&F}$ and $QCH_{T\&F}$ and ± 20% for $LOQ_{T\&F}$, respectively.

Matrix effects were investigated using six blank plasma samples from healthy volunteers. For both total and free teicoplanin, plasma samples and ultrapure water were prepared for analysis but after extraction, 180 µL supernatant was spiked with 20 µL of low and high concentration calibrator stock solution. Matrix effects were evaluated by calculating the matrix factor (MF), which is the ratio of the peak area of teicoplanin or internal standard measured in plasma to the peak area of teicoplanin (MF_{teico}) or internal standard (MF_{IS}) measured in water. Matrix effects were assumed to be negligible when MF_{teico}/MF_{IS} was within ±15 % for both low and high concentration sets. For calculations, the mean of the obtained peak areas was used.

Storage and freeze/thaw stability was studied using QCL_T, QCM_T and QCH_T samples. For storage stability, these QC samples were stored at 4 °C for 1, 4, 7 and 14 days and the obtained total and free concentrations were compared to the same samples which were stored at -80 °C. Freeze/thaw stability was evaluated by comparing obtained total and free concentrations of QCL_T, QCM_T and QCH_T after three freeze/thaw cycles (at -80 °C) with those which were stored at -80 °C. Stability was assumed when total and free concentrations were within ± 15 % of the concentrations found for QC samples which were stored at -80 °C.

3.5.3.10 Effect of blood collection tube type: spiked plasma samples

Plasma (K_2EDTA and lithium heparin) and serum (SST) from healthy volunteers (all n = 6) were collected to study the possible effect of different blood tube types on total and free teicoplanin concentrations as well as on the %PB. These plasma and serum samples were spiked after thawing with either a low (final concentration: 11.2 mg/L) or high (final concentration: 56 mg/L) concentration of teicoplanin. Subsequently, these samples were incubated for 30 min at RT prior to sample preparation. Concentrations were determined using a calibration curve in a pool of blank K_2EDTA plasma (total teicoplanin) or blank K_2EDTA plasma ultrafiltrate (free teicoplanin) only.

3.5.3.11 Method comparison: patient and spiked samples

For method comparison, the same left-over plasma (K_2EDTA and lithium heparin) and serum samples (n = 41) from patients who received teicoplanin were used. K_2EDTA plasma samples (n = 7) from healthy subjects which were spiked with teicoplanin were included as well. Each sample was stored at -80 °C immediately after collection until batch analysis was performed. Total teicoplanin concentrations were assayed by a validated homogeneous particle-enhanced turbidimetric (*i.e.* quantitative microsphere system, QMS® teicoplanin) immunoassay [58,60] on an Architect c4000 instrument (Abott, IL, USA) as well as by the UHPLC-HRMS method described here. Plasma and serum samples were quantified using a calibration curve prepared in the corresponding matrix. Subsequently, the obtained concentrations were compared by Passing-Bablok regression and a Bland-Altman plot, using Medcalc software (Medcalc BVBA, Ostend, Belgium).

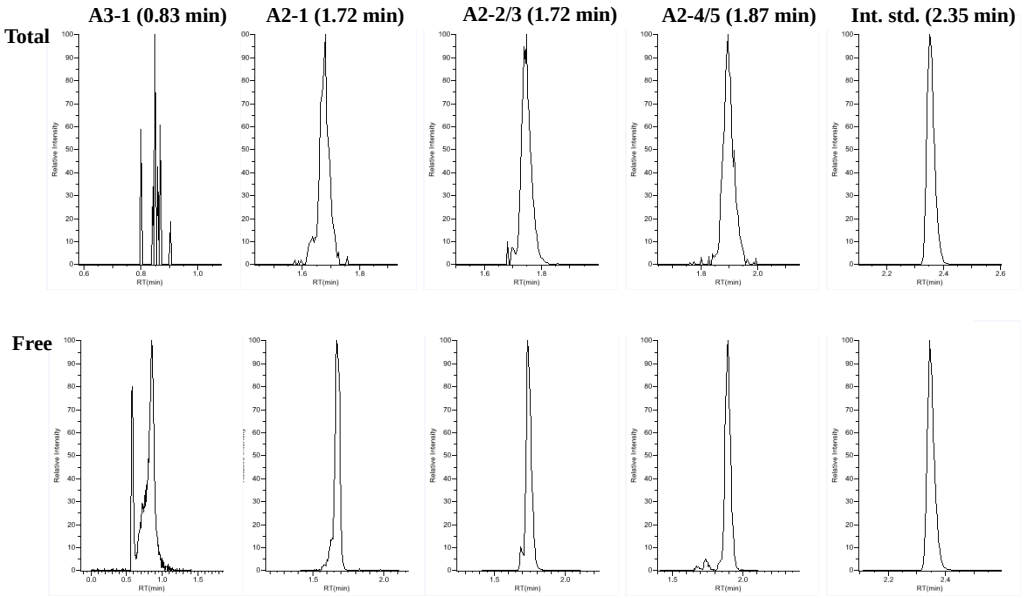
3.5.4 Results

3.5.4.1 Chromatography

The described UHPLC-HRMS method was used to separate teicoplanin compounds A3-1, A2-1, A2-2 & A2-3 (pair of isomers) and A2-4 & A2-5 (pair of isomers) for the quantification of total and free teicoplanin. For both assays (*i.e.* total and free), the same separation procedure was used and the total run times were 4.5 min. The retention time of each teicoplanin compound is provided in Table 3-6. For total teicoplanin assay, compounds were detected (underlined m/z

values in Table 3-6) in full scan whereas for free teicoplanin, three scan segments were introduced and 5 mass isotopes (m/z 1 to 5 in Table 3-6) were monitored to enhance sensitivity. A representative chromatogram for each compound is shown in Figure 3-5.

Figure 3-5 Representative chromatograms obtained from the highest calibrator standard for total and free teicoplanin compounds (A3-1, A2-1, A2-2 & A2-3 and A2-4 & A2-5), as well as for the internal standard (int. std.).



3.5.4.2 Equilibrium dialysis versus ultrafiltration

Teicoplanin recovery was 112.3 ± 12.1 % in ED analyses, and 23.1 ± 2.7 % and 79.5 ± 8.0 % in UF analyses using Amicon and Centrifree filters, respectively. Total (C_T) and free (C_F) teicoplanin concentrations as obtained by ED (37 °C) and UF (RT and 37 °C, Centrifree filters only) in individually spiked plasma samples were used to calculate the %PB of teicoplanin in each sample (Table 3-7). The overall mean value of C_T, C_F and %PB is also given in Table 3-7. The overall mean %PB was 93.6 ± 0.4 %, 94.1 ± 1.3 % and 95.8 ± 1.1 % as obtained by ED (37 °C), UF (37 °C) and UF (RT), respectively.

3.5.4.3 %PB in patient samples

The mean total and free teicoplanin concentrations as determined in 41 patient samples were 23.9 mg/L (range 6.5 to 53.3 mg/L) and 2.7 mg/L (range 0.2 to 6.7 mg/L), respectively. The mean %PB of teicoplanin as determined in 41 patients was 87.7 % and ranged from 79.6 to 95.4 %.

Table 3-7 Total (C_T) and free (C_F) teicoplanin concentrations and the corresponding percentage protein binding (%PB) as obtained by equilibrium dialysis at 37 °C and ultrafiltration at 37 °C and room temperature (RT) in blank plasma samples, which were spiked with a low, medium or high concentration of teicoplanin.

	C_T (mg/L)	C_F (mg/L)			%PB (%)		
		ED (37 °C)	UF (37 °C)	UF (RT)	ED (37 °C)	UF (37 °C)	UF (RT)
Low	**7[a]**						
Sample 1	7.5	0.5	0.5	0.4	93.1	93.0	94.4
Sample 2	6.7	0.4	0.5	0.4	93.9	92.2	94.4
Medium	**70[a]**						
Sample 3	76.5	4.7	4.3	3.0	93.9	94.3	96.1
Sample 4	73.7	5.1	3.3	2.6	94.1	95.6	96.5
High	**140[a]**						
Sample 5	124.8	8.6	6.8	4.3	93.1	94.6	96.6
Sample 6	149.7	9.8	7.3	4.5	93.5	95.1	97.0
Overall					**93.6 ± 0.4**	**94.1 ± 1.3**	**95.8 ± 1.1**

[a] Theoretical values. Values are expressed as mean ± standard deviation.

3.5.4.4 Assay validation

Selectivity. No detectable interferences were found in the individual blank EDTA plasma samples for teicoplanin. The QC samples showed no interference with the internal standard and in both types of analyses, a small peak was detected at the m/z ratio of the internal standard but its response was < 5%. However, in a number of patient samples which were collected in different blood tubes, an interfering peak for A2-4 & A2-5 detection was observed at m/z = 946.793. Therefore, we selected m/z = 948.295 for interference-free A2-4 & A2-5 detection (Table 3-6). Thus, the method was selective for all teicoplanin compounds and the internal standard at these settings.

Carry-over. No detectable peaks were found in individual blank plasma samples which were analyzed after the highest calibrator standard.

LOQ. No peaks were detected in blank samples and the LOQ was 1.4 and 0.3 mg/L for total and free teicoplanin, respectively.

Calibration curves. Appropriate calibration regressions and weightings were selected based on a recently described mathematical model [62,63]. For total teicoplanin, a quadratic curve was used with weighting factor x^{-2} ($R^2 = 0.99 \pm 0.004$; $n = 5$), whereas for free teicoplanin, a linear curve was used with weighting factor x^{-2} ($R^2 = 0.99 \pm 0.008$; $n = 5$). Table 3-8 summarizes the precision of the back calculated concentrations and the accuracy to their corresponding theoretical value. Except for a high bias for calibrator standard 3 of free teicoplanin (15.4 %), all other calibrator standards had a precision < 15 % and a bias within ± 15 % of the nominal value. Therefore, the calibration curves were accepted.

Precision and accuracy. Data on both within-run and between-run precision and accuracy are tabulated Table 3-9. The within-run precision was 5.2 % and 4.4 % at LOQ level and ranged from 6.3 to 12.9 % and from 3.1 to 6.6 % at QC levels, for total and free teicoplanin, respectively. The between-run precision was 1.7 and 15.4 % at LOQ level and ranged from 1.8 to 7.6 % and from 4.8 to 11.5 % at QC levels, for total and free teicoplanin, respectively. Within-run accuracy values ranged from -10.4 to 9.6 %. Between-run accuracy ranged from -10.5 to 11.7 %.

Table 3-8 Calibration curve information.

	Theoretical value (mg/L)	Calculated value (mg/L)	Precision (CV%)	Accuracy (%Bias)
Total teicoplanin				
Cal 1	1.4	1.4 ± 0.1	5.5	0.7
Cal 2	2.8	2.8 ± 0.2	8.0	-1.3
Cal 3	7.0	6.9 ± 1.0	14.7	-1.7
Cal 4	28.0	30.5 ± 2.9	9.4	8.8
Cal 5	70.0	62.5 ± 5.6	9.0	-10.7
Cal 6	105.0	108.5 ± 10.0	9.2	3.1
Cal 7	140.0	141.1 ± 16.3	11.6	0.8
Free teicoplanin				
Cal 1	0.3	0.3 ± 0.01	4.4	-4.9
Cal 2	1.4	1.6 ± 0.2	12.5	13.0
Cal 3	2.8	2.4 ± 0.2	7.2	-15.4
Cal 4	7.0	7.4 ± 0.3	4.0	6.3
Cal 5	10.5	10.1 ± 0.8	7.6	3.7
Cal 6	14.0	14.1 ± 0.8	5.5	1.0

Calculated values are expressed as mean ± standard deviation; CV%: coefficient of variation; n = 5 for both total and free teicoplanin.

Matrix effects. Peak areas of total and free teicoplanin as obtained in spiked extracts of blank plasma, blank plasma ultrafiltrate and ultrapure water with a low and a high concentration are provided in Table 3-10, together with peak areas of the internal standard. Except for low total teicoplanin, MF_{Teico}/MF_{IS} was within ± 15 % for each concentration set.

Storage and freeze/thaw stability. Each QC sample was stable over a period of 14 consecutive days when stored at 4 °C as compared to those which were stored at -80 °C, for both total and free teicoplanin (all within ± 15 %). In addition, three freeze/thaw cycles of each QC sample had no effect on the stability of teicoplanin (all within ± 15 %).

.

Table 3-9 Assay precision and accuracy.

		Within-run			Between-run		
	Theoretical value (mg/L)	**Calculated value (mg/L)**	**Precision (CV%)**	**Accuracy (%Bias)**	**Calculated value (mg/L)**	**Precision (CV%)**	**Accuracy (%Bias)**
Total teicoplanin							
$LLOQ_T$	1.4	1.4 ± 0.1	5.4	1.2	1.4 ± 0.04	2.8	1.9
QCL_T	7.0	7.4 ± 0.9	12.6	6.0	7.6 ± 0.4	5.1	7.9
QCM_T	70.0	77.7 ± 4.8	6.2	10.9	77.5 ± 7.5	9.7	10.7
QCH_T	140.0	131.8 ± 9.3	7.1	-5.9	155.5 ± 9.4	6.0	11.1
Free teicoplanin							
$LLOQ_F$	0.3	0.3 ± 0.01	4.4	-4.9	0.3 ± 0.04	15.4	-10.5
QCL_F	0.9	0.9 ± 0.04	4.1	-3.6	0.9 ± 0.04	4.8	-3.7
QCM_F	5.6	5.0 ± 0.2	3.1	-10.4	5.0 ± 0.3	5.3	-9.9
QCH_F	12.6	11.9 ± 0.8	6.6	-5.7	12.3 ± 1.4	11.5	-2.4

Calculated values are expressed as mean ± standard deviation; CV%: coefficient of variation; n = 5 (within-run) or n = 4 (between-run: after 1, 4, 7 and 14 days) for both total and free teicoplanin.

Table 3-10 Peak areas of teicoplanin (Teico) and internal standard (Int. std.) as measured in plasma and water, to study the matrix effect.

	Plasma	**Water**	**MF_{Teico} or MF_{IS}**	**MF_{Teico}/MF_{IS}**
Total teicoplanin				
Teico (L)	3781232 ± 427582	3400519 ± 243514	1.11	1.25
Int. std. (L)	64898049 ± 3951204	72712426 ± 6139386	0.89	
Teico (H)	65404115 ± 12736450	63246396 ± 8181394	1.03	1.13
Int. std. (H)	66193315 ± 3720803	72488916 ± 2533207	0.91	
Free teicoplanin				
Teico (L)	7453429 ± 936331	7424363 ± 1197619	1.00	1.02
Int. std. (L)	123306400 ± 73059824	125210396 ± 10686508	0.98	
Teico (H)	89200239 ± 5338651	104190165 ± 24525044	0.86	0.92
Int. std. (H)	124503213 ± 12100533	133167832 ± 43036386	0.93	

Peak areas are expressed as mean ± standard deviation; L: low concentration; H: high concentration; all n = 6

3.5.4.5 Effect of blood collection tubes: spiked plasma samples

Total and free teicoplanin concentrations as determined in spiked plasma (K_2EDTA and lithium heparin) and serum (SST) samples from healthy donors are summarized in Table 3-11. For both low and high concentration sets in K_2EDTA plasma, total teicoplanin concentrations corresponded to their nominal values. Because of the limited number of repetitions (n = 3), values were not statistically compared, but a trend of higher total concentrations when determined in lithium heparin plasma and in serum samples was observed as compared to those in K_2EDTA plasma. This is due to a higher peak area of teicoplanin and not to a lower peak area of the internal standard (data not shown). However, no clear difference was found in free teicoplanin concentration amongst the different matrices. Consequently, a small increase in %PB was calculated in lithium heparin and serum samples as compared to in K_2EDTA samples.

Table 3-11 Effect of blood tube type on the total (C_T) and free (C_F) teicoplanin concentration and the corresponding percentage protein binding (%PB).

	C_T (mg/L)	C_F (mg/L)	%PB (%)
Low	**11.2**[a]		
K_2EDTA plasma	11.3 ± 1.1	1.0 ± 0.1	91.5 ± 0.5
Lithium heparin plasma	15.5 ± 1.4	1.2 ± 0.1	92.3 ± 0.6
SST serum	21.2 ± 3.5	1.1 ± 0.1	94.7 ± 1.1
High	**56.0**[a]		
K_2EDTA plasma	54.8 ± 4.4	4.9 ± 1.4	90.8 ± 3.3
Lithium heparin plasma	67.0 ± 8.9	4.7 ± 0.3	92.9 ± 1.1
SST serum	81.6 ± 10.1	4.3 ± 0.9	94.7 ± 0.7

[a] Theoretical values; calculated values are expressed as mean ± standard deviation; n = 3 for both low and high concentrations of teicoplanin.

3.5.4.6 Method comparison: patient and spiked samples

Total teicoplanin concentrations determined by QMS® teicoplanin analysis and the UHPLC-HRMS method described here were compared by Passing-Bablok regression and a Bland-Altman plot, provided in Figure 3-6 and Figure 3-7 respectively. For Passing-Bablok regression, the correlation coefficient between both methods was 0.82 (95 % confidence interval: 0.70 – 0.90). Total teicoplanin concentrations were slightly higher when measured with the newly developed UHPLC-HRMS method as compared to the QMS® method. Bland-Altman analysis revealed a mean bias of 3.2 % between both methods and therefore, the here described UHPLC-HRMS method was accepted.

Figure 3-6 Passing-Bablok regression for method comparison of total teicoplanin between the QMS® teicoplanin assay and the newly developed UHPLC-HRMS method.

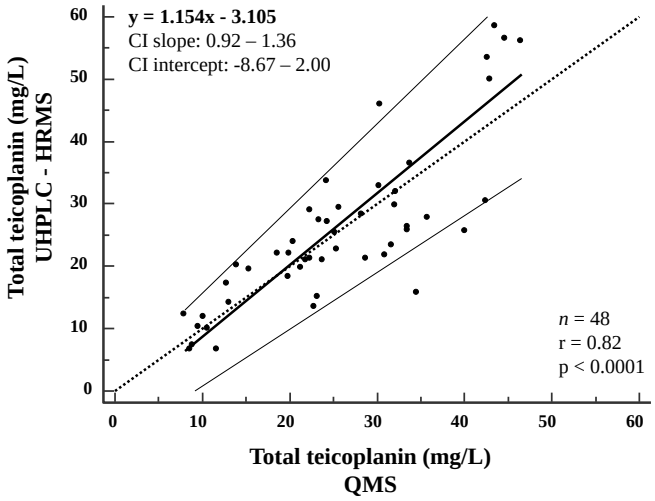
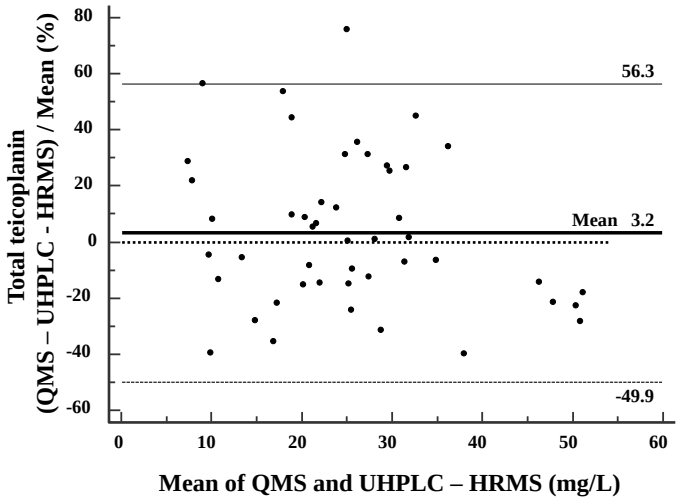


Figure 3-7 Bland-Altman plot for method comparison of total teicoplanin between the QMS® teicoplanin assay and the newly developed UHPLC-HRMS method.



3.5.5 Discussion

A novel UHPLC-HRMS method for the quantification of total and free teicoplanin in K_2EDTA plasma samples was developed and validated according to EMA guidelines. The here described method requires only 200 µL of plasma to quantify both the total and free teicoplanin concentration, which is much lower as compared to methods described elsewhere (700 µL in [38] and > 1 mL in [46]), and covers the complete therapeutic window of teicoplanin: ranging from 1.4 – 140 and 0.3 – 14 mg/L for total and free teicoplanin, respectively. In literature, only a limited number of papers discussed the analysis of both total and free teicoplanin [38,46,56]. To the best of our knowledge, only one paper described the analysis of total teicoplanin (*i.e.*

A2-1, A2-2 & A2-3 and A2-4 & A2-5) using the same HRMS instrument [60]. However, in our method, four teicoplanin compounds (*i.e.* A3-1, A2-1, A2-2 & A2-3 and A2-4 & A2-5) were separated by UHPLC resulting in a total run time of only 4.5 min, which is half the time as presented by Mueller *et al.* where HPLC was used [60].

This method was selective for both teicoplanin and the internal standard in K_2EDTA samples. The precision and accuracy of the total and free assay were within the requirements of the EMA guidelines, for both within-run and between-run analyses. *In house* prepared QC samples were all stable over a period of 14 consecutive days and three freeze/thaw cycles.

A signal increase of teicoplanin was observed in spiked lithium heparin samples and in spiked serum samples as compared to spiked K_2EDTA plasma samples. This effect could possibly be explained by a difference in ionization efficiency of the teicoplanin compounds between the different tested sample types, as previously found for other drug substances [64,65]. In a number of patient samples, an unknown interfering compound was present for A2-4 & A2-5 detection at m/z = 946.793. Therefore, we selected a less abundant isotope at m/z = 948.295 for interference-free A2-4 & A2-5 detection. In this study, we were not able to elucidate the origin of this interfering compound and to the best of our knowledge, this is the first time that the presence of an interfering compound for teicoplanin A2-4 & A2-5 is reported. Therefore, we can only formulate a number of hypotheses which should be studied in future research, including the presence of concomitantly administered drugs, heparins as anticoagulant in lithium heparin blood tubes or compounds migrating from the separator gel in serum blood tubes (probably others than those used here in spiking experiments), as previously reported for other compounds [66,67].

It should also be mentioned that no deuterated teicoplanin was used as internal standard. Consequently, there was no compound-specific correction for sample loss during sample preparation, sample injection or sample ionization. However, the method described here showed an acceptable precision and the obtained results were accurate. Therefore, the usage of expensive deuterated teicoplanin seemed to be unnecessary in our method, as well as in other mass spectrometry based teicoplanin assays [59–61].

Free teicoplanin concentrations were obtained by ultrafiltration at 37 °C and were close to the values obtained by equilibrium dialysis, which was also performed at 37 °C to mimic the human body's temperature. Ultrafiltration at room temperature should be avoided since this leads to a small decrease (not statistically tested because n = 3) in free teicoplanin concentrations,

resulting in a slightly higher %PB. Furthermore, ultrafiltration should not be carried out at 4 °C because of the apparent higher %PB of teicoplanin [46]. The use of Amicon filters is not recommended either because of their low recovery for teicoplanin. Calibrator levels for free teicoplanin calibration were also ultrafiltered after dilutions in ultrafiltrate to correct for the NSA to the Centrifree filters, which was around 20 %. The %PB (90 – 95 %) reported here for teicoplanin in spiked plasma samples was in good agreement with *in vivo* values reported in the literature where ultrafiltration was performed at 37 °C, but where no evidence for the accuracy of the obtained free fractions (e.g. by comparison with ED) was presented [38, 39]. In addition, the %PB of teicoplanin was found constant in the studied physiological concentration range, *i.e.* from 7 to 140 mg/L. The *in vivo* %PB values as obtained in patient samples was 87.7 % (range: 79.6 – 95.4 %), pointing out the large inter-patient variability, which was also reported in [38, 39].

In routine analyses, the QMS® teicoplanin assay is often used for quantification of total teicoplanin because of its short turn-around time as no sample preparation is required. This QMS® teicoplanin assay may, however, suffer from non-specific interferences since it is hypothesized that the used antibodies can also interact with compounds other than teicoplanin [60]. Furthermore, the LOQ of this QMS® teicoplanin assay is 10 mg/L, which is assumed to be the minimum for total teicoplanin through concentrations [50,51]. Consequently, free teicoplanin, *i.e.* the biologically active form, is usually < 10 mg/L and cannot be detected in a reliable way. Therefore, the major advantages of the present method, as compared to the QMS® teicoplanin assay, are its higher selectivity and sensitivity as well as its higher precision [68]. The correlation (r = 0.82) between the newly developed UHPLC-HRMS method and the QMS® teicoplanin assay was moderate, but in line with the correlation reported by Mueller *et al.* who used the same MS instrument, but only for total teicoplanin analysis purposes [60]. Not only K_2EDTA plasma samples, but also serum en lithium heparin samples were used for method comparison. Hence, our UHPLC-HRMS method was developed and validated using K_2EDTA plasma samples, but is also applicable for analysis of serum samples and even lithium heparin samples, but then, calibration curves should be constructed in the corresponding matrix.

This new UHPLC-HRMS method can be useful in TDM. Although there is no consensus on the therapeutic value for free teicoplanin so far, it would be of additional value since the free fraction is biologically active and thus of therapeutic relevance. Nowadays, this free fraction is often estimated by a general percentage of around 10 %, despite the large inter-patient variability as demonstrated in this paper and in [38,39]. With our method, the target attainment

of free teicoplanin can be determined in a more reliable way as compared to the estimation of 10%.

3.5.6 Conclusion

In conclusion, a novel highly sensitive and fast UHPLC-HRMS method was developed and validated according to EMA guidelines for the quantification of total and free teicoplanin in human K_2EDTA plasma samples. Furthermore, our method is applicable for interference-free total and free teicoplanin analysis in serum and lithium heparin samples. Amongst others, this method can be useful in therapeutic drug monitoring, especially when knowledge of the free teicoplanin concentration is important.

3.5.7 Acknowledgements

This project was supported by The Research Foundation – Flanders (FWO Vlaanderen, Project Number G0A4614N). The authors acknowledge Mieke Carlier for the collection of patient samples and the Toxicology Lab staff for their assistance.

3.6 References

1. Merck Company Amicon Ultra-0.5mL Centrifugal Filters for DNA and Protein Purification and Concentration. http://www.merckmillipore.com/BE/en/product/Amicon-Ultra-0.5nbspmL-Centrifugal-Filters-for-DNA-and-Protein-Purification-and-Concentration,MM_NF-C82301?ReferrerURL=http%3A%2F%2Fwww.emdmillipore.com%2FUS%2Fen%2Fproduct%2FAmicon-Ultra-0.5nbspmL-Centrifugal-F. Accessed 19 Dec 2017

2. Vuignier K, Schappler J, Veuthey J-L, Carrupt P-A, Martel S (2010) Drug-protein binding: a critical review of analytical tools. Anal Bioanal Chem 398:53–66. doi: 10.1007/s00216-010-3737-1

3. Nilsson LB (2013) The bioanalytical challenge of determining unbound concentration and protein binding for drugs. Bioanalysis 5:3033–50. doi: 10.4155/bio.13.274

4. Stove V, Coene L, Carlier M, Waele JJ De, Fiers T, Verstraete AG (2015) Measuring Unbound Versus Total Vancomycin Concentrations in Serum and Plasma : Methodological Issues and Relevance. Ther Drug Monit 37:180–187.

5. Deltombe O, Dhondt A, Van Biesen W, Glorieux G, Eloot S (2017) Effect of sample temperature, pH, and matrix on the percentage protein binding of protein-bound uraemic toxins. Anal Methods 9:1935–1940. doi: 10.1039/C7AY00054E

6. Committee for Medicinal Products for Human Use (CHMP) (2011) Guideline on bioanalytical method validation - European Medicines Agency.

7. Kadavil J (2013) Guidance for Industry Bioanalytical Method Validation Guidance for Industry Bioanalytical Method Validation Center for Veterinary Medicine (CVM) Contains Nonbinding Recommendations. Commun Staff 20855:240–276.

8. Banker MJ, Clark TH (2008) Plasma / Serum Protein Binding Determinations. Curr Drug Metab 9:854–859.

9. Kratzer A, Liebchen U, Schleibinger M, Kees MG, Kees F (2014) Determination of free vancomycin, ceftriaxone, cefazolin and ertapenem in plasma by ultrafiltration: Impact of experimental conditions. J Chromatogr B Anal Technol Biomed Life Sci 961:97–102. doi: 10.1016/j.jchromb.2014.05.021

10. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clarck W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jörres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W (2003) Review on uremic toxins: Classification, concentration and interindividual variability. Am J Soc Nephrol 63:1934–1943. doi: 10.1046/j.1523-1755.2003.00924.x

11. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A (2012) Normal and Pathologic Concentrations of Uremic Toxins. J Am Soc Nephrol 23:1258–1270. doi: 10.1681/ASN.2011121175

12. Schepers E, Meert N, Glorieux G, Goeman J, Van der Eycken J, Vanholder R (2007) P-cresylsulphate, the main in vivo metabolite of p-cresol, activates leucocyte free radical production. Nephrol Dial Transplant 22:592–596. doi: 10.1093/ndt/gfl584

13. Adijiang A, Goto S, Uramoto S, Nishijima F, Niwa T (2008) Indoxyl sulphate promotes aortic calcification with expression of osteoblast-specific proteins in hypertensive rats. Nephrol Dial Transplant 23:1892–901. doi: 10.1093/ndt/gfm861

14. Barreto FC, Barreto D V, Liabeuf S, Meert N, Glorieux G, Temmar M, Choukroun G, Vanholder R, Massy ZA (2009) Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. Clin J Am Soc Nephrol 4:1551–1558. doi: 10.2215/CJN.03980609

15. Meijers BKI, Van kerckhoven S, Verbeke K, Dehaen W, Vanrenterghem Y, Hoylaerts MF, Evenepoel P (2009) The Uremic Retention Solute p-Cresyl Sulfate and Markers of Endothelial Damage. Am J Kidney Dis 54:891–901. doi: 10.1053/j.ajkd.2009.04.022

16. Liabeuf S, Barreto D V., Barreto FC, Meert N, Glorieux G, Schepers E, Temmar M, Choukroun G, Vanholder R, Massy Z a. (2010) Free p-cresylsulphate is a predictor of mortality in patients at different stages of chronic kidney disease. Nephrol Dial Transplant 25:1183–1191. doi: 10.1093/ndt/gfp592

17. Jourde-Chiche N, Dou L, Cerini C, Dignat-George F, Brunet P (2011) Vascular Incompetence in Dialysis Patients-Protein-Bound Uremic Toxins and Endothelial Dysfunction. Semin Dial 24:327–337. doi: 10.1111/j.1525-139X.2011.00925.x

18. Sirich TL, Aronov PA, Plummer NS, Hostetter TH, Meyer TW (2013) Numerous protein-bound solutes are cleared by the kidney with high efficiency. Kidney Int 84:585–90. doi: 10.1038/ki.2013.154

19. Vanholder R, Schepers E, Pletinck A, Nagler E, Glorieux G (2014) The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review. J Am Soc Nephrol 1–11. doi: 10.1681/ASN.2013101062

20. Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339–347. doi: 10.1053/ajkd.2002.34518

21. Eloot S, Van Biesen W, Axelsen M, Glorieux G, Pedersen RS, Heaf JG (2015) Protein-bound solute removal during extended multipass versus standard hemodialysis. BMC Nephrol 16:57. doi: 10.1186/s12882-015-0056-y

22. Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S (2015) Exploring Protein Binding of Uremic Toxins in Patients with Different Stages of Chronic Kidney Disease and during Hemodialysis. Toxins (Basel) 7:3933–3946. doi: 10.3390/toxins7103933

23. Arund J, Luman M, Uhlin F, Tanner R, Fridolin I (2016) Is Fluorescence Valid to Monitor Removal of Protein Bound Uremic Solutes in Dialysis? PLoS One 11:e0156541. doi: 10.1371/journal.pone.0156541

24. de Loor H, Bammens B, Evenepoel P, De Preter V, Verbeke K (2005) Gas chromatographic-mass spectrometric analysis for measurement of p-cresol and its conjugated metabolites in uremic and normal serum. Clin Chem 51:1535–8. doi: 10.1373/clinchem.2005.050781

25. Meert N, Schepers E, Glorieux G, Van Landschoot M, Goeman JL, Waterloos MA, Dhondt A, Van Der Eycken J, Vanholder R (2012) Novel method for simultaneous determination of p-cresylsulphate and p-cresylglucuronide: Clinical data and pathophysiological implications. Nephrol Dial Transplant 27:2388–2396. doi: 10.1093/ndt/gfr672

26. Itoh Y, Ezawa A, Kikuchi K, Tsuruta Y, Niwa T (2012) Protein-bound uremic toxins in hemodialysis patients measured by liquid chromatography/tandem mass spectrometry

and their effects on endothelial ROS production. Anal Bioanal Chem 403:1841–1850. doi: 10.1007/s00216-012-5929-3

27. Boelaert J, Lynen F, Glorieux G, Eloot S, Van Landschoot M, Waterloos MA, Sandra P, Vanholder R (2013) A novel UPLC-MS-MS method for simultaneous determination of seven uremic retention toxins with cardiovascular relevance in chronic kidney disease patients. Anal Bioanal Chem 405:1937–1947. doi: 10.1007/s00216-012-6636-9

28. de Loor H, Poesen R, De Leger W, Dehaen W, Augustijns P, Evenepoel P, Meijers B (2016) A liquid chromatography – tandem mass spectrometry method to measure a selected panel of uremic retention solutes derived from endogenous and colonic microbial metabolism. Anal Chim Acta 936:149–156. doi: 10.1016/j.aca.2016.06.057

29. Hinderling PH, Hartmann D (2005) The pH dependency of the binding of drugs to plasma proteins in man. Ther Drug Monit 27:71–85. doi: 10.1097/00007691-200502000-00014

30. Kochansky CJ, McMasters DR, Lu P, Koeplinger K a., Kerr HH, Shou M, Korzekwa KR (2008) Impact of pH on plasma protein binding in equilibrium dialysis. Mol Pharm 5:438–448. doi: 10.1021/mp800004s

31. Brørs O, Jacobsen S (1985) pH lability in serum during equilibrium dialysis. Br J Clin Pharmacol 20:85–88.

32. Kodama H, Kodama Y, Itokazu N, Shinozawa S, Kanemaru R, Sugimoto T (2001) Effect of temperature on serum protein binding characteristics of phenytoin in monotherapy paediatric patients with epilepsy. J Clin Pharm Ther 26:175–179. doi: 10.1046/j.1365-2710.2001.00340.x

33. Bertuzzi A, Mingrone G, Gandolfi A, Greco A V., Ringoir S, Vanholder R (1997) Binding of indole-3-acetic acid to human serum albumin and competition with L-tryptophan. Clin Chim Acta 265:183–192.

34. Sakai T, Yamasaki K, Sako T, Kragh-Hansen U, Suenaga A, Otagiri M (2001) Interaction mechanism between indoxyl sulfate, a typical uremic toxin bound to site II, and ligands bound to site I of human serum albumin. Pharm Res 18:520–524. doi: 10.1023/A:1011014629551

35. Viaene L, Annaert P, De Loor H, Poesen R, Evenepoel P, Meijers B (2013) Albumin is the main plasma binding protein for indoxyl sulfate and p-cresyl sulfate. Biopharm Drug Dispos 34:165–175. doi: 10.1002/bdd.1834

36. Ohshima T, Hasegawa T, Johno I, Kitazawa S (1989) Variations in protein binding of drugs in plasma and serum. Clin Chem 35:1722–1725.

37. Roberts JA, Pea F, Lipman J (2012) The Clinical Relevance of Plasma Protein Binding Changes. Clin Pharmacokinet 52:1–8. doi: 10.1007/s40262-012-0018-5

38. Roberts JA, Stove V, De Waele JJ, Sipinkoski B, McWhinney B, Ungerer JPJ, Akova M, Bassetti M, Dimopoulos G, Kaukonen KM, Koulenti D, Martin C, Montravers P, Rello J, Rhodes A, Starr T, Wallis SC, Lipman J (2014) Variability in protein binding of teicoplanin and achievement of therapeutic drug monitoring targets in critically ill patients: Lessons from the DALI Study. Int J Antimicrob Agents 43:423–430. doi: 10.1016/j.ijantimicag.2014.01.023

39. Yano R, Nakamura T, Tsukamoto H, Igarashi T, Goto N, Wakiya Y, Masada M (2007) Variability in teicoplanin protein binding and its prediction using serum albumin concentrations. Ther Drug Monit 29:399–403. doi: 10.1097/FTD.0b013e3180690755

40. Niekerk L Van, Lipman J, Roberts JA (2015) Albumin concentration significantly impacts on free teicoplanin plasma concentrations in non-critically ill patients with chronic bone sepsis ☪. Int J Antimicrob Agents 45:647–651. doi: 10.1016/j.ijantimicag.2015.01.015

41. Somma S, Gastaldo L, Corti A (1984) Teicoplanin, a New Antibiotic from Actinoplanes teichomyceticus nov. sp. Antimicrob Agents Chemother 26:917–923.

42. Wilson APR, Griineberg RN, Neub H (1994) A critical review of the dosage of teicoplanin in Europe and the USA. Int J Antimicrob Agents 4:S1–S30.

43. Ziglam HM, Finch RG (2001) Limitations of presently available glycopeptides in the treatment of Gram- positive infection. Clin Microbiol Infect 7:53–65. doi: 10.1046/j.1469-0691.2001.00059.x

44. Matsumoto K, Watanabe E, Kanazawa N, Fukamizu T, Shigemi A, Yokoyama Y, Ikawa K (2016) Pharmacokinetic/pharmacodynamic analysis of teicoplanin in patients with MRSA infections. Clin Pharmacol Adv Appl 8:15–18. doi: 10.2147/CPAA.S96143

45. Borghi A, Antonini P, Zanol M, Ferrari P, Zerilli LF, Lancini GC (1988) Isolation and structure determination of two new analogs of teicoplanin, a glycopeptide antibiotic. J Antibiot (Tokyo) 42:361–366.

46. Dykhuizen RS, Harvey G, Stephenson N, Nathwani D, Gould IM (1995) Protein binding and serum bactericidal activities of vancomycin and teicoplanin. Antimicrob Agents Chemother 39:1842–1847. doi: 10.1128/AAC.39.8.1842

47. Peter A, Wilson R (2000) Clinical Pharmacokinetics of Teicoplanin. Clin Pharmacokinet 39:167–183.

48. Svetitsky S, Leibovici L, Paul M (2009) Comparative Efficacy and Safety of Vancomycin versus Teicoplanin: Systematic Review and Meta-Analysis. Antimicrob Agents Chemother 53:4069–4079. doi: 10.1128/AAC.00341-09

49. Cavalcanti AB, Goncalves AR, Almeida CS, Gomes DB, Silva E (2010) Teicoplanin versus vancomycin for proven or suspected infection. Cochrane Database Syst Rev. doi: 10.1002/14651858.CD007022

50. Tobin CM, Lovering AM, Sweeney E, Macgowan AP (2010) Analyses of teicoplanin concentrations from 1994 to 2006 from a UK assay service. J Antimicrob Chemother 65:2155–2157. doi: 10.1093/jac/dkq266

51. Gould IM, Cauda R, Esposito S, Gudiol F, Mazzei T, Garau J (2011) Management of serious meticillin-resistant Staphylococcus aureus infections: what are the limits? Int J Antimicrob Agents 37:202–209. doi: 10.1016/j.ijantimicag.2010.10.030

52. Roberts JA, Norris R, Paterson DL, Martin JH (2012) Therapeutic drug monitoring of antimicrobials. Br J Clin Pharmacol 73:27–36. doi: 10.1111/j.1365-2125.2011.04080.x

53. MacGowan A, White L, Reeves D, Harding I (1996) Retrospective review of serum teicoplanin concentrations in clinical trials and their relationship to clinical outcome. J Infect Chemother 2:197–208. doi: 10.1007/BF02355116

54. Harding I, MacGowan AP, White LO, Darley ES, Reed V (2000) Teicoplanin therapy for Staphylococcus aureus septicaemia: relationship between pre-dose serum concentrations and outcome. J Antimicrob Chemother 45:835–41.

55. Rybak MJ, Bailey EM, Reddyt VN (1991) Clinical Evaluation of Teicoplanin Fluorescence Polarization Immunoassay. Antimicrob Agents Chemother 35:1586–1590.

56. Urakami T, Maiguma T, Kaji H, Kondo S, Teshima D (2008) Analysis using fluorescence polarization immunoassay for unbound teicoplanin concentration in serum. J Clin Pharm Ther 33:357–363. doi: 10.1111/j.1365-2710.2008.00923.x

57. Yu L, Zhong M, Wei Y (2010) Direct Fluorescence Polarization Assay for the Detection of Glycopeptide Antibiotics. Anal Chem 82:7044–7048. doi: 10.1021/ac100543e

58. Dailly E, Fraissinet F, Deslandes G, Bouquié R, Jolliet P (2013) Evaluation of the QMS® Teicoplanin Immunoassay (ThermoFisher Scientific) on Cobas® 8000 System (Roche Diagnostics) and Comparison to Fluorescence Polarization Immunoassay for the Determination of Teicoplanin Concentrations in Human Plasma. J Clin Lab Anal 27:96–98. doi: 10.1002/jcla.21567

59. Tsai I-L, Sun H-Y, Chen G-Y, Lin S-W, Kuo C-H (2013) Simultaneous quantification of antimicrobial agents for multidrug-resistant bacterial infections in human plasma by ultra-high-pressure liquid chromatography-tandem mass spectrometry. Talanta 116:593–603. doi: 10.1016/j.talanta.2013.07.043

60. Mueller DM, Von Eckardstein A, Saleh L (2014) Quantification of teicoplanin in plasma by LC-MS with online sample clean-up and comparison with QMS (R) assay. Clin Chem Lab Med 52:879–887. doi: 10.1515/cclm-2013-0974

61. Kim K-Y, Cho S-H, Song Y-H, Nam M-S, Kim C-W (2016) Direct injection LC–MS/MS method for the determination of teicoplanin in human plasma. J Chromatogr B 1008:125–131. doi: 10.1016/j.jchromb.2015.11.037

62. Desharnais B, Camirand-Lemyre F, Mireault P, Skinner CD (2017) Procedure for the selection and validation of a calibration model I-description and application. J Anal Toxicol 41:261–268. doi: 10.1093/jat/bkx001

63. Desharnais B, Camirand-Lemyre F, Mireault P, Skinner CD (2017) Procedure for the selection and validation of a calibration model II-theoretical basis. J Anal Toxicol 41:269–276. doi: 10.1093/jat/bkx002

64. Chin C, Zhang ZP, Karnes HT (2004) A study of matrix effects on an LC/MS/MS assay for olanzapine and desmethyl olanzapine. J Pharm Biomed Anal 35:1149–1167. doi: 10.1016/J.JPBA.2004.01.005

65. Barri T, Dragsted LO (2013) UPLC-ESI-QTOF/MS and multivariate data analysis for blood plasma and serum metabolomics: Effect of experimental artefacts and anticoagulant. Anal Chim Acta 768:118–128. doi: 10.1016/J.ACA.2013.01.015

66. Shi RZ, van Rossum HH, Bowen RAR (2012) Serum testosterone quantitation by liquid chromatography-tandem mass spectrometry: Interference from blood collection tubes. Clin Biochem 45:1706–1709. doi: 10.1016/J.CLINBIOCHEM.2012.08.008

67. Bowen RAR, Remaley AT (2014) Interferences from blood collection tube components on clinical chemistry assays. Biochem Medica 24:31–44. doi: 10.11613/BM.2014.006

68. Bourget P, Lesne-Hulin A, Sertin A, Maillot A, Alaya M, Martin C (1997) Fluorescence polarization immunoassay: does it always represent a reliable method to monitor treatment with teicoplanin? Comparison with data obtained by high-performance liquid chromatography. Int J Pharm 146:4797–7.

Chapter 4

Protein binding of uremic toxins in patients with chronic kidney disease

4.1 Introduction

In addition to small water-soluble solutes (MW < 500 Da) and middle molecules (MW > 500 Da), protein-bound uremic toxins (PBUTs, mostly MW < 500 Da) are accumulated in patients with chronic kidney disease [1,2]. Different PBUTs are known to contribute to the increased inflammatory and cardiovascular morbidity in patients with CKD [3–11]. In addition, in patients on hemodialysis (HD), removal of these PBUTs is hampered because of their binding to plasma proteins, such that only the free fraction is able to be transported in a passive way through the pores of the dialyzer membrane [12–14].

Both aspects, *i.e.* the toxicity of PBUTs and their hampered removal during HD, have been studied for almost three decennia, but are still the topic of ongoing research. One problem here is that most of the performed studies only focused on the total PBUT concentration while, in analogy to the pharmacokinetics of drug compounds, the free fraction of PBUTs is assumed to be biologically active. Hence, the percentage protein binding (%PB) might be an important parameter to study in uremia.

Therefore, in this section, the protein binding of a selected panel of PBUTs is described in patients with different stages of CKD as well as during a HD session.

4.2 Exploring protein binding of uremic toxins in patients with different stages of chronic kidney disease and during hemodialysis

Based on: Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S (2015) Exploring Protein Binding of Uremic Toxins in Patients with Different Stages of Chronic Kidney Disease and during Hemodialysis. Toxins (Basel) 7:3933–3946. doi: 10.3390/toxins7103933

4.2.1 Abstract

As protein binding of uremic toxins is not well understood, neither in chronic kidney disease (CKD) progression, nor during a hemodialysis (HD) session, we studied protein binding in two cross-sectional studies. Ninety-five CKD 2 to 5 patients and ten stable hemodialysis patients were included. Blood samples were taken either during the routine ambulatory visit (CKD patients) or from blood inlet and outlet line during dialysis (HD patients). Total (C_T) and free concentrations were determined of *p*-cresyl glucuronide (*p*CG), hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS), and their percentage protein binding (%PB) was calculated. In CKD patients, %PB/C_T resulted in a positive correlation (all $p < 0.001$) with creatinine clearance for all five uremic toxins. In HD patients, %PB was increased after 120 min of dialysis for HA and at the dialysis end for the stronger (IAA) and the highly-bound (IS and *p*CS) solutes. During one passage through the dialyzer at 120 min, %PB was increased for HA (borderline), IAA, IS and *p*CS. These findings explain why protein-bound solutes are difficult to remove by dialysis: a combination of the fact that (i) only the free fraction can pass the filter and (ii) the equilibrium, as it was pre-dialysis, cannot be maintained during the dialysis session, as it is continuously disturbed.

4.2.2 Introduction

Uremic syndrome is characterized by the retention of a large number of compounds, which in healthy persons are excreted by the kidneys. Some of those retention solutes interact negatively with biological functions and are called uremic toxins. These toxins are classified into three groups: the free small water-soluble solutes (molecular weight (MW) < 500 Da), the middle molecules (MW > 500 Da) and the protein-bound solutes [1,2]. The latter solutes have a protein binding ranging from around 10 % (e.g., *p*-cresyl glucuronide) to near 100 % (e.g., *p*-cresyl sulfate). Many of these protein-bound substances are known to exert toxicity in a direct or

indirect way [8,10,15]. However, in our current understanding, toxicity is only exerted by the free concentration and not by the protein-bound concentration.

Since the extent of protein binding depends on the solute concentration, the protein concentration, the protein-solute affinity constant and the presence or absence of competing solutes [16], each protein-bound uremic toxin binds to the specific proteins to a variable degree. Structural changes of proteins can also alter the extent of protein binding, and therefore, it can be hypothesized that the degree of protein binding also changes in individual patients with the progression of their chronic kidney disease (CKD), as post-translational modifications (oxidation, carbamylation and glycosylation as the most relevant processes) of proteins increase as CKD progresses [17]. During hemodialysis (HD), only the free fraction can be removed, such that the overall dialyzer clearance depends, apart from blood and dialysate flow and dialyzer characteristics, on the free toxin concentration and on the speed of equilibration between bound and free fractions. Free fractions, and with it dialyzer clearance, can be increased, for example, by the presence of competing ligands in the serum, like sodium octanoate [18,19], or by infusing hypertonic saline at the dialyzer inlet [20–22]. The mechanisms of protein binding are, however, not well understood.

In this study, we evaluated the percentage protein binding at different stages of CKD (*i.e.* Stages 2 to 5), during a hemodialysis session in dialysis patients and in healthy controls. This information might be useful in the development of new removal strategies aiming at the optimization of dialysis.

4.2.3 Materials and methods

To calculate the percentage protein binding in CKD patients, data were taken from a cross-sectional study including 95 patients with confirmed diagnosis of CKD Stages 2 to 5. To unravel changes in protein binding in the hemodialyzer, as well as during the course of a hemodialysis session, data were taken from a second cross-sectional study including 10 stable HD patients. Data from 10 healthy controls with normal renal function were also collected to cover the range (healthy-CKD-HD) of free and total toxin concentrations, as well as for protein binding.

4.2.3.1 Patients and sampling protocol

CKD patients: 95 CKD patients (CKD Stages 2 to 5) were included from Amiens University Hospital (France), in whom concentrations of uremic retention solutes were evaluated for their relation to clinical outcomes [5,7,23]. These patients were older than 40 years and had a confirmed diagnosis of CKD (creatinine clearance below 90 mL/min, calculated in the aforementioned study according to the Cockcroft-Gault formula [24]). Exclusion criteria were the presence of chronic inflammatory disease, atrial fibrillation, complete heart block, abdominal aorta aneurysm, aortic and/or femoral artery prosthesis, primary hyperparathyroidism, kidney transplantation, treated with dialysis and any acute cardiovascular event in the 3 months before screening for inclusion. Blood samples were taken in the morning on the occasion of a visit at the outpatient clinic.

HD patients: 10 stable hemodialysis patients were included from Ghent University Hospital. Exclusion criteria were active infection, pregnancy, unstable condition, vascular access problems and age below 18 years. During the experimental session at midweek, conventional two needle/lumen HD was performed for 240 min using high-flux dialyzers: FX800 (n = 6) (Fresenius Medical Care, Bad Homburg, Germany), Evodial (n = 1) (Gambro, Lund, Sweden), Xenium 210 (n = 1) (Baxter, Dearfield, IL, USA), Phylter HF17G (n = 1) and Phylter HF17SD (n = 1) (Bellco, Mirandola, Italy) in a diffusive mode. Blood and dialysate flows were set at 300 and 700 mL/min, respectively, while ultrafiltration rates were set according to the needs of the patients. Nine patients had a well-functioning fistula and one patient a Bard Optiflow central venous catheter (Bard, Covengton, GA, USA) as vascular access. Residual renal function was calculated as the arithmetic mean of the creatinine and urea clearance, calculated from the interdialytic urine collection (volume and concentration) and blood concentrations at the start and end of the interdialytic period [25]. During the experimental session, blood samples were collected at the start of the session from the vascular access and from the inlet blood line after 60 and 120 min, and immediately after discontinuation of the dialysis session (at 240 min). Blood samples were also collected from the outlet blood line after 120 min since the start of the dialysis session.

Both studies were approved by the local ethical committees (Comité de protection des Personnes Nord-Ouest II, CHU Amiens, Amiens, France, 06H3 for CKD patients, and Ghent University Hospital, Ghent, Belgium, UZG 2008/128 for healthy controls and 2008/081 for HD

patients), performed in accordance with the principles of the Declaration of Helsinki, and all patients gave their written informed consent.

Healthy Controls: Data from 10 healthy volunteers with normal renal function were collected at Ghent University Hospital. Subjects who were smoking, had an infection, were pregnant or on medication were excluded.

4.2.3.2 Laboratory

All blood samples were immediately centrifuged after sampling and serum was stored at -80 °C until batch analysis.

Concentrations of protein-bound uremic toxins were determined by reversed-phase high performance liquid chromatography (RP-HPLC), as described earlier [26,27]. The solutes analyzed were *p*-cresyl glucuronide (*p*CG, MW: 284.3 Da), hippuric acid (HA, MW: 179.2 Da), indole-3-acetic acid (IAA, MW: 174.2 Da), indoxyl sulfate (IS, MW: 212.2 Da) and *p*-cresyl sulfate (*p*CS, MW: 187.2 Da). To determine the total concentration, serum samples were first deproteinized by heat denaturation prior to HPLC analysis [12]. HA was analyzed by UV detection at 254 nm, whereas *p*CG and *p*CS ($\lambda_{exc.}$ = 265 nm, λ_{em} = 290 nm) and IAA and IS ($\lambda_{exc.}$ = 280 nm, λ_{em} = 340 nm) were determined by fluorescence detection [12,26]. To obtain free fractions, untreated serum samples were filtered through a Centrifree filter device (Millipore Billerica, MA, USA) prior to heating [12]. Albumin levels in serum from CKD patients were assayed in a biochemistry laboratory using standard autoanalyzer techniques (the Modular IIP system, Roche Diagnostics, Basel, Switzerland) [5,7,23]. In HD patients, total protein concentration in serum was analyzed according to standard methods (Biuret reaction).

4.2.3.3 Calculations

Percentage protein binding (%PB) was calculated from the measured total (C_T) and free (C_F) concentrations as:

$$\%PB = \left(1 - \frac{C_F}{C_T}\right) \times 100\ \% \qquad \text{(Eq. 4-1)}$$

The reduction ratio (RR) for the free and total concentration in HD patients was determined from the concentration at the start of the dialysis session (C_{pre}) and after time t = 60, 120 and 240 min (C_t) as:

$$RR = \left(1 - \frac{C_t}{C_{pre}}\right) \times 100\ \% \qquad \text{(Eq. 4-2)}$$

4.2.3.4 Statistical Analysis

Statistical evaluation was performed with SPSS Statistics 22 (2013, Armonk, NY, USA). Data were checked for normality. As most numeric data were not normally distributed, data were expressed as the median [25th percentile (pct); 75th pct]. To compare independent categorical data, Fisher's exact test was performed. Differences between more than two groups of unpaired data (CKD data) were checked with a Kruskal–Wallis test (with multiple comparisons and Bonferroni correction). Paired comparisons (HD data) between more than two groups were made with a Friedman test (with multiple comparisons and Bonferroni correction). To evaluate the difference between two paired groups, the Wilcoxon signed-rank test was applied. Spearman's rho test was performed to check correlation (all presented p- and R-values are Spearman's rho values, unless stated otherwise). An univariate general linear model was used with covariates (C_T, DM and albumin concentration) to check any improvements of the correlation between creatinine clearance and %PB. A linear regression procedure was used to check differences in regression coefficients between two groups (reduction ratio). $P < 0.05$ was considered significant, and all tests were two-tailed.

4.2.4 Results and discussion

4.2.4.1 Patient characteristics

Table 4-1, Table 4-2 and Table 4-3 present the demographic and clinical characteristics of the 95 CKD patients, the 10 HD patients and the 10 healthy controls with normal renal function, respectively.

Besides differences in creatinine clearance among the CKD stages, no dissimilarities were observed among the different CKD stages for age, gender, body mass index (BMI), diabetes mellitus (DM) and albumin concentration.

Table 4-1 Demographic and clinical characteristics of the CKD patients.

Characteristics	CKD patients				
	CKD 2 to 5	**CKD 2**	**CKD 3**	**CKD 4**	**CKD 5**
Number, *n* (%)	95 (100)	11 (11.5)	37 (39)	37 (39)	10 (10.5)
Age (years)	69 [59;76]	62 [59;71]	74 [61;77]	69 [55;74]	79 [60;83]
Male gender, *n* (%)	59 (62)	9 (82)	24 (65)	22 (60)	4 (40)
BMI (kg/m^2)	29 [25;32]	27 [21;29]	29 [25;32]	29 [26;34]	25 [23;30]
DM, *n* (%)	45 (47)	4 (36)	19 (51)	18 (49)	4 (40)
Albumin (g/L)	39 [35;44]	42 [37;47]	38 [35;42]	41 [35;44]	33 [28;39]
Creatinine clearance[1] (mL/min)	32 [20;49]	67 [63;71]	45 [35;51]	22 [19;25]°,+	11 [9;13]°,+

CKD: chronic kidney disease; BMI: body mass index; DM: diabetes mellitus. Median [25th percentile (pct); 75th pct]. ° $p < 0.05$ *versus* CKD 2; $^{+}$ $p < 0.05$ *versus* CKD 3. [1] Creatinine clearance calculated according to the Cockcroft-Gault formula.

Table 4-2 Demographic and clinical characteristics of the HD patients.

Characteristics	HD patients
Age (years)	72 [61;78]
Male gender, *n* (%)	8 (80)
Ultrafiltration (mL/min)	4.8 [3.5;8.9]
BMI (kg/m^2)	28 [25;28]
DM, *n* (%)	5 (50)
Total protein (g/L)	60 [58;67]
Creatinine clearance (mL/min)	2.6 [0.0;4.1]

HD: hemodialysis. Median [25th pct; 75th pct].

Table 4-3 Demographic and clinical characteristics of the healthy controls.

Characteristics	Healthy controls
Age (years)	40 [33;57]
Male gender, *n* (%)	4 (40)
BMI (kg/m^2)	23 [19;27]

Median [25th pct; 75th pct].

The median percentage protein binding (%PB), the free and total concentration of *p*-cresyl glucuronide (*p*CG), hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) are shown in Table 4-4 for an increasing degree of kidney failure, *i.e.* consecutively for the healthy controls, the CKD patients and the HD patients. Since the three groups differ in origin, nutrition and hospital center, they were not statistically compared to each other.

For the healthy controls, the free and total concentration of *p*CG were below the limit of quantification (LOQ), and the %PB could not be calculated. The median %PB was 34 % (HA), 83 % (IAA), 84 % (IS) and 94 % (*p*CS).

In the different stages of CKD, the free and total concentrations of the weakly-bound solutes *p*CG and HA were only increased in CKD 5 patients, and for the stronger bound solutes IAA, IS and *p*CS, already at CKD Stage 4.

Table 4-4 Percentage protein binding (%PB), free (C_F) and total (C_T) concentration of protein-bound solutes in healthy controls with normal renal function, in CKD and HD patients (pre-dialysis).

Uremic Toxin	%PB or Concentration	Healthy controls	CKD				HD
			CKD 2	CKD 3	CKD 4	CKD 5	
	%PB	-	7 [3;24]	10 [6;14]	9 [6;16]	8 [5;20]	12 [9;19]
***p*CG**	C_F (mg/dL)	<LOQ	0.03 [0.03;0.04]	0.03 [0.03;0.04]	0.04 [0.03;0.07]	0.20 [0.07;0.25]$^{\circ,+}$	0.31 [0.22;0.85]
	C_T (mg/dL)	<LOQ	0.03 [0.03;0.05]	0.04 [0.03;0.04]	0.05 [0.03;0.08]	0.25 [0.08;0.28]$^{\circ,+,\#}$	0.35 [0.25;0.99]
	%PB	34 [22;39]	38 [34;42]	38 [34;43]	38 [35;44]	43 [36;45]	39 [32;54]
HA	C_F (mg/dL)	0.13 [0.10;0.16]	0.23 [0.18;0.38]	0.20 [0.18;0.32]	0.30 [0.21;0.42]	0.54 [0.34;0.69]$^{+}$	1.59 [0.73;3.30]
	C_T (mg/dL)	0.17 [0.11;0.25]	0.39 [0.32;0.58]	0.33 [0.27;0.53]	0.51 [0.33;0.53]	0.93 [0.51;1.25]$^{+}$	2.41 [1.57;5.42]
	%PB	83 [78;84]	60 [56;66]	67 [61;75]	66 [61;72]	68 [65;71]	69 [63;80]
IAA	C_F (mg/dL)	0.01 [0.01;0.01]	0.02 [0.02;0.02]	0.03 [0.02;0.03]	0.03 [0.03;0.03]$^{\circ,(+)}$	0.03 [0.03;0.04]$^{\circ,+}$	0.07 [0.04;0.11]
	C_T (mg/dL)	0.04 [0.03;0.05]	0.06 [0.05;0.07]	0.08 [0.06;0.12]	0.09 [0.07;0.11]$^{\circ}$	0.11 [0.10;0.15]$^{\circ}$	0.19 [0.13;0.33]
	%PB	84 [77;88]	77 [71;83]	86 [80;90]	89 [87;92]$^{\circ,+}$	92 [90;95]$^{\circ,+}$	93 [90;95]
IS	C_F (mg/dL)	0.02 [0.01;0.02]	0.03 [0.03;0.03]	0.03 [0.03;0.03]	0.04 [0.03;0.04]$^{\circ,+}$	0.06 [0.03;0.08]$^{\circ,+}$	0.08 [0.04;0.21]
	C_T (mg/dL)	0.10 [0.06;0.14]	0.16 [0.11;0.18]	0.23 [0.16;0.35]	0.36 [0.28;0.55]$^{\circ,+}$	0.79 [0.31;1.50]$^{\circ,+}$	1.40 [0.69;2.18]
	%PB	94 [87;96]	93 [89;96]	97 [96;97]$^{\circ}$	96 [95;97]	94 [93;95]$^{+}$	95 [93;97]
***p*CS**	C_F (mg/dL)	0.02 [0.01;0.02]	0.05 [0.03;0.07]	0.04 [0.02;0.05]	0.05 [0.03;0.12]	0.21 [0.12;0.31]$^{+,\#}$	0.06 [0.04;0.10]
	C_T (mg/dL)	0.31 [0.08;0.47]	0.47 [0.38;0.70]	0.95 [0.59;1.37]	1.19 [0.65;2.52]$^{\circ}$	3.29 [1.52;4.47]$^{\circ,+}$	2.06 [1.14;2.87]

*p*CG: *p*-cresyl glucuronide; HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate. LOQ: limit of quantification; Median [25th pct; 75th pct].
$^{\circ}$ $p < 0.05$ *versus* CKD 2; $^{+}$ $p < 0.05$ *versus* CKD 3; $^{\#}$ $p < 0.05$ *versus* CKD 4; (+) $p = 0.061$.

The median percentage protein binding was in the range 7 % to 8 % (*p*CG), 38 % to 43 % (HA), 60 % to 68 % (IAA), 77 % to 92 % (IS) and 93 % to 94 % (*p*CS). For the highly-bound IS, %PB was increased in CKD 4 and 5 patients with respect to CKD 2 and 3 patients, while for *p*CS, %PB showed some variation, but without a clear trend. The median %PB in the HD patients was 12 % (*p*CG), 39 % (HA), 69 % (IAA), 93 % (IS) and 95 % (*p*CS).

4.2.4.2 CKD patients

Considering the 95 CKD patients, only %PB of IS showed an (inverse) correlation with creatinine clearance ($R = -0.64$; $p < 0.001$) (Figure 4-5 in 4.2.7 Supplementary figures and Table 4-5). Normalizing %PB for total toxin concentration, however, resulted in a positive correlation (all $p < 0.001$) with creatinine clearance for all five uremic toxins (Figure 4-1).

Figure 4-1 Percentage protein binding (%PB) normalized for total toxin concentration (C_T) *versus* creatinine clearance of CKD patients for: (A) *p*-cresyl glucuronide; (B) hippuric acid; (C) indole-3-acetic acid; (D) indoxyl sulfate; and (E) *p*-cresyl sulfate.

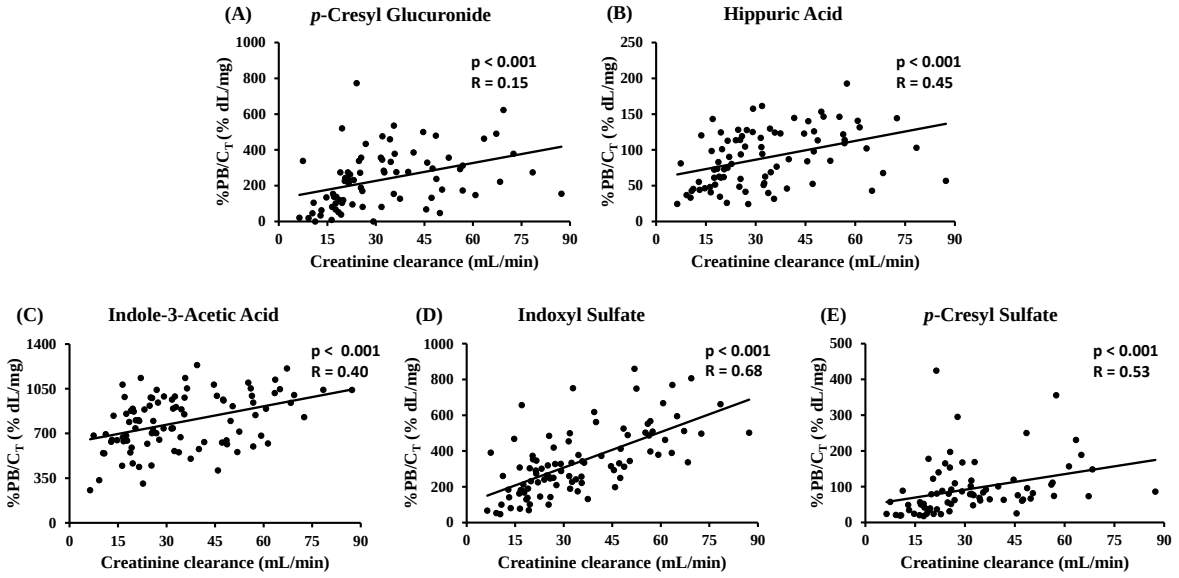


Table 4-5 shows the p- and R^2-values of the correlation test between %PB and creatinine clearance in the 95 CKD patients. The influence of added covariates C_T, diabetes mellitus and the albumin concentration was checked, as shown in Table 4-5. The total toxin concentration improved the model (all $p < 0.05$), except for *p*CG (Table 4-5). This result is in line with the correlations found in Figure 4-1. When diabetes mellitus was added to the model, only an improvement was found for IAA and IS (Table 4-5). However, a lower R^2-value was given compared to C_T. Including albumin concentration did not improve the correlation between %PB and creatinine clearance (Table 4-5), neither did it correlate with %PB (Figure 4-6 in

4.2.7 Supplementary figures). Therefore, the albumin concentration (in this range) did not have an influence on the %PB, as already published for other compounds in the literature [27,28].

Table 4-5 p- and R^2-values of the correlations between %PB and creatinine clearance (CrCl) for *p*CG, HA, IAA, IS and *p*CS and the influence of added covariates total toxin concentration (C_T), diabetes mellitus (DM) and albumin concentration in CKD patients.

Uremic Toxin	%PB *versus* CrCl		Covariates					
			C_T		DM		Albumin	
	p	R^2	p	R^2	p	R^2	p	R^2
***p*CG**	0.65	-	0.081	-	0.288	-	0.91	-
HA	0.40	-	**0.008**	0.23	0.623	-	0.49	-
IAA	0.18	-	**0.002**	0.71	**0.028**	0.03	0.85	-
IS	**< 0.001**	0.41	**< 0.001**	0.67	**< 0.001**	0.32	0.77	-
***p*CS**	0.12	-	**0.004**	0.16	0.843	-	0.62	-

P < 0.05 is indicated in bold. R^2-values only shown in the case of significant p.

It is known that post-translational modifications of proteins increase as CKD progresses, with carbamylation, oxidation, glycosylation and guanidinylation as the most relevant processes [17,29]. Whether our result is influenced by these structural changes in the proteins or whether this is the result of competitive binding, leading to an enhanced free toxin concentration, remains unclear. Anyway, the presented results are in line with those for highly (around 90%) protein-bound drugs, like valproic acid or phenytoin, also showing a decreased protein binding in patients with renal failure [30,31].

4.2.4.3 HD patients

In hemodialysis patients, median pre-dialysis %PB was 12 % (*p*CG), 39 % (HA), 69 % (IAA), 93 % (IS) and 95 % (*p*CS). Figure 4-2 and Table 4-6 show %PB determined at the inlet of the dialyzer, at the start (0 min) of the hemodialysis session and after 60, 120 and 240 min.

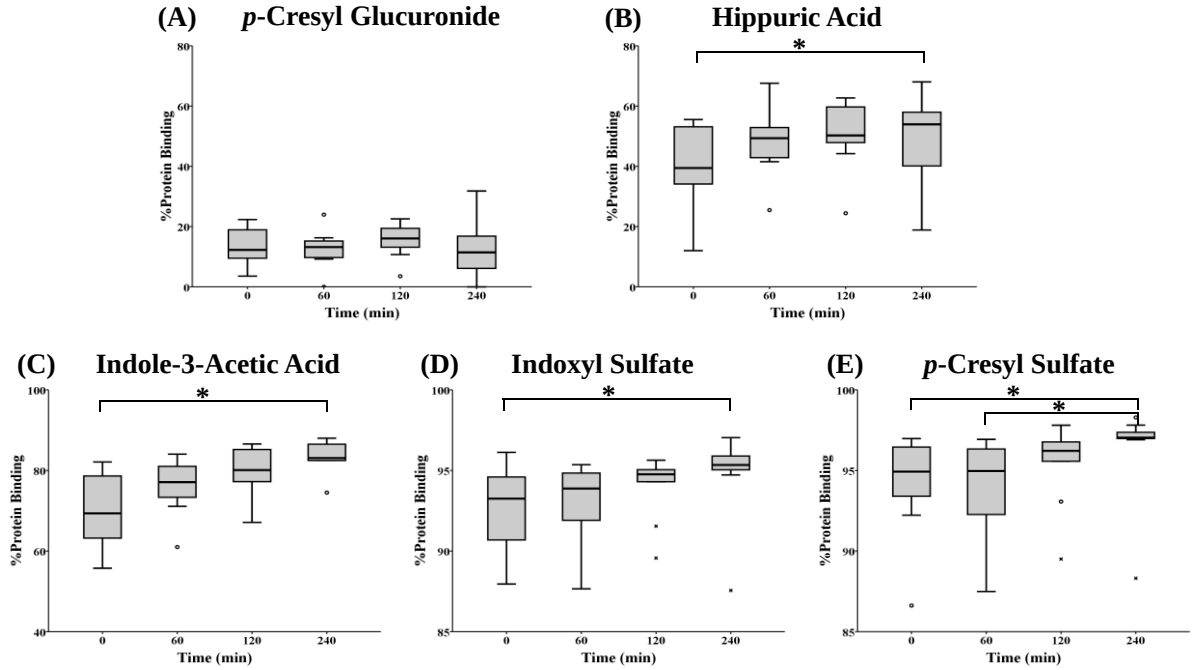
The total and free toxin concentrations were decreased from 120 min on for *p*CG, HA and *p*CS. For IAA, only the total concentration decreased from 120 min on and for IS, only the free concentration (Table 4-6). No differences in %PB were observed during the HD session for the weakly-bound *p*CG. For HA, %PB was increased after 120 and 240 min *versus* HD start, while for the stronger (IAA) and highly-bound (IS and *p*CS) solutes, this increase was only significant after 240 min (Figure 4-2 and Table 4-6).

Table 4-6 Percentage protein binding, free and total concentration of protein-bound solutes in HD patients at different time points during an HD session.

Uremic Toxin	%PB or Concentration	0 min	60 min	120 min	240 min
***p*CG**	%PB	12 [9;19]	13 [10;15]	10 [13;20]	11[5;19]
	C_F (mg/dL)	0.31 [0.22;0.85]	0.14 [0.11;0.44]	0.11 [0.07;0.29]°	0.08 [0.04;0.16]°,+
	C_T (mg/dL)	0.35[0.25;0.99]	0.17 [0.12;0.45]	0.14 [0.08;0.36]°	0.10 [0.05;0.19]°,+
HA	%PB	39 [32;54]	49 [43;54]	50 [47;60]°	54 [39;58]°
	C_F (mg/dL)	1.59 [0.73;3.30]	0.91 [0.41;1.84]	0.70 [0.44;1.37]°	0.42 [0.32;0.82]°,+
	C_T (mg/dL)	2.41 [1.57;5.42]	1.42 [0.99;3.52]	1.37 [0.97;2.55]°	0.95 [0.61;1.49]°,+
IAA	%PB	69 [63;80]	77 [72;82]	80 [77;86]	83 [78;87]°
	C_F (mg/dL)	0.07 [0.04;0.11]	0.04 [0.02;0.08]	0.03 [0.02;0.06]	0.03 [0.02;0.07]°
	C_T (mg/dL)	0.19 [0.13;0.33]	0.14 [0.10;0.26]	0.13 [0.10;0.23]°	0.10 [0.08;0.16]°,+
IS	%PB	93 [90;95]	94 [91;95]	95 [94;95]	95 [95;96]°
	C_F (mg/dL)	0.08 [0.04;0.21]	0.06 [0.04;0.16]	0.06 [0.03;0.12]°	0.03 [0.02;0.07]°,+
	C_T (mg/dL)	1.40 [0.69;2.18]	1.14 [0.60;1.97]	1.06 [0.56;1.75]	0.72 [0.48;1.47]°,+
***p*CS**	%PB	95[93;97]	95 [92;96]	96 [95;97]	97 [97;97]°,+
	C_F (mg/dL)	0.12[0.10;0.24]	0.13 [0.09;0.19]	0.09 [0.07;0.14]°	0.06 [0.04;0.10]°,+
	C_T (mg/dL)	2.76[1.75;4.25]	2.46 [1.45;3.70]	2.27 [1.32;3.41]°	2.06 [1.14;2.87]°,+

° $p < 0.05$ *versus* 0 min; $^{+}p < 0.05$ *versus* 60 min. Median [25th pct; 75th pct].

Figure 4-2 Percentage protein binding at different time points during a hemodialysis (HD) session for: (A) *p*-cresyl glucuronide; (B) hippuric acid; (C) indole-3-acetic acid; (D) indoxyl sulfate; and (E) *p*-cresyl sulfate.

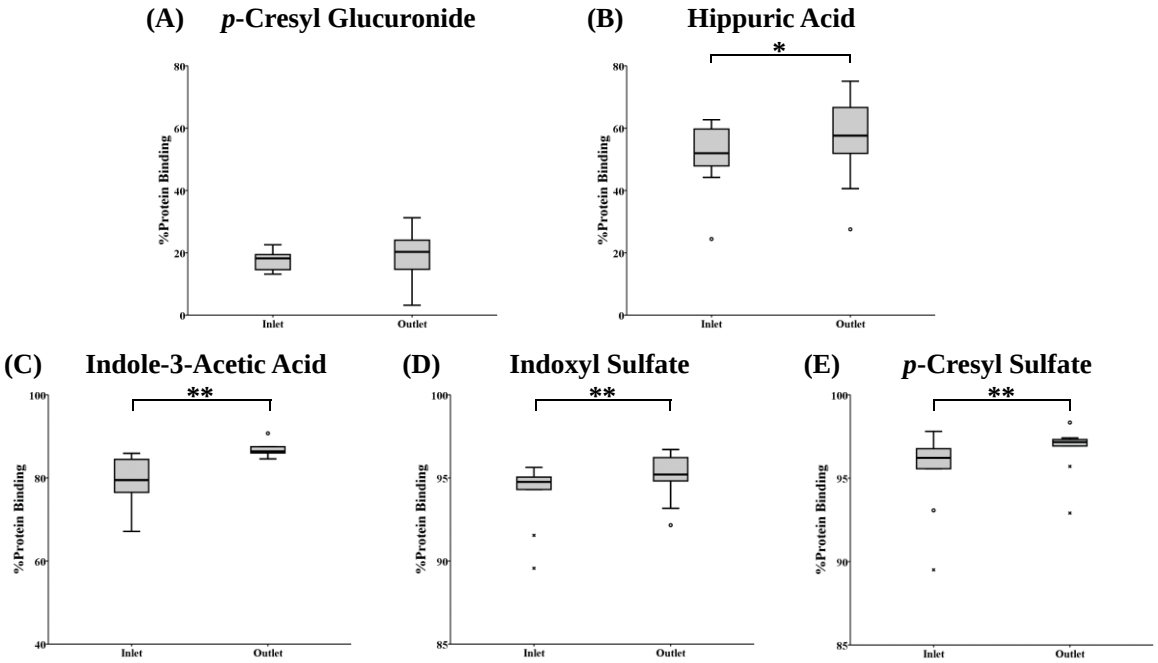


* $p < 0.05$; o: outlier; x: extreme.

Changes in percentage protein binding from the dialyzer inlet towards the outlet as measured at 120 min after dialysis start are depicted in Figure 4-3. For the weakly-bound *p*CG, passage through the dialyzer did not influence the percentage binding. For HA, the %PB was increased with a borderline significance ($p = 0.066$). The percentage protein binding for the stronger

(IAA) and the highly-bound (IS and *p*CS) solutes was significantly increased from dialyzer inlet to outlet at 120 min.

Figure 4-3 Percentage protein binding at the dialyzer inlet versus outlet after 120 min since dialysis start for: (A) *p*-cresyl glucuronide; (B) hippuric acid; (C) indole-3-acetic acid; (D) indoxyl sulfate; and (E) *p*-cresyl sulfate.



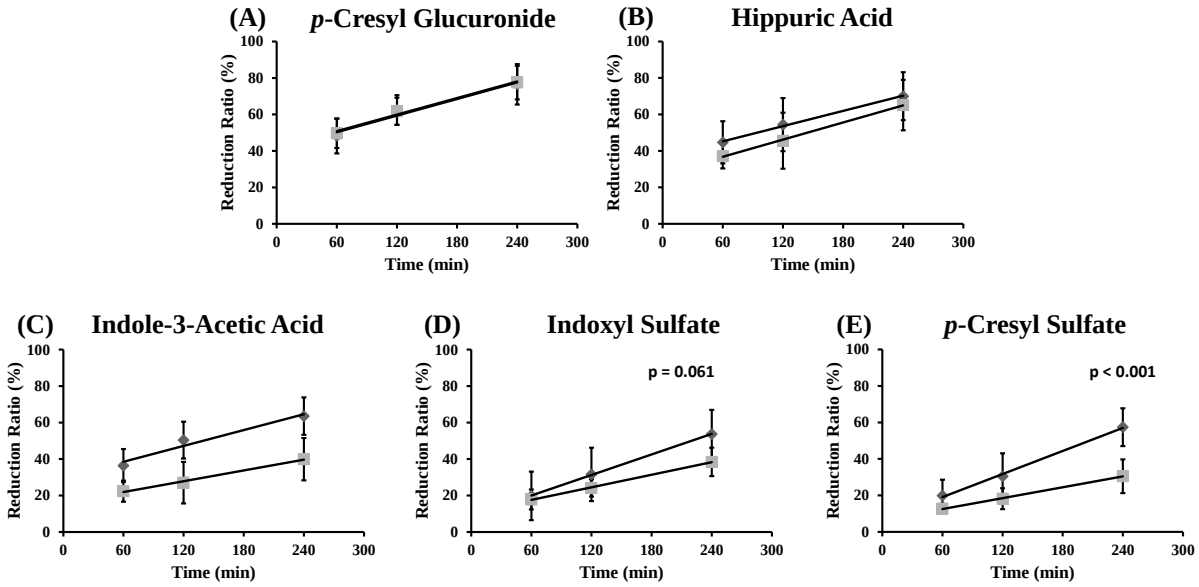
** p < 0.05 *versus* the inlet; * p = 0.066 *versus* the inlet; o: outlier; x: extreme.

This study is the first to demonstrate that the percentage protein binding for stronger bound solutes changes during dialysis, both within the dialyzer itself during a single passage and within a patient (*i.e.* during the course of the dialysis session). The influence of hemoconcentration on these observations was checked by a correlation test between the change in %PB and the change in total protein concentration at the inlet and outlet of the dialyzer, but no significant correlation was found (Figure 4-7 in 4.2.7 Supplementary figures). Neither can this be explained by changes in pH, since we only observed limited pH changes during the course of a dialysis session.

A possible explanation might be found in the hypothesis that the physicochemical bond of the toxin with its protein is strong. As a consequence, the equilibrium reaction is too slow to restore the free toxin concentration within the time frame of a single passage through the dialyzer and even within the time frame of a dialysis session once the pool of available pre-dialysis free fraction has been removed. Therefore, we calculated the reduction ratio (RR) for both total and free toxin concentration to check this hypothesis. The RR is graphically presented in Figure 4-4

for *p*CG, HA, IAA, IS and *p*CS at 60, 120 and 240 min since the start of the dialysis session. The slopes of free and total RR are borderline significant for IS ($p = 0.061$) and significantly different for *p*CS ($p < 0.001$).

Figure 4-4 Reduction ratio for total (▭) and free (◆) toxin concentration at different time points during an HD session



(A) *p*-cresylglucuronide (free: $y = 0.15x + 41$; $R^2 = 0.96$; total: $y = 0.15x + 42$; $R^2 = 0.99$);
(B) hippuric acid (free: $y = 0.14x + 37$; $R^2 = 1.00$; total: $y = 0.16x + 27$; $R^2 = 1.00$);
(C) indole-3-acetic acid (free: $y = 0.14x + 30$; $R^2 = 0.96$; total: $y = 0.10x + 16$; $R^2 = 0.99$);
(D) indoxyl sulfate (free: $y = 0.19x + 8.7$; $R^2 = 1.00$; total: $y = 0.11x + 11$; $R^2 = 1.00$); and
(E) *p*-cresylsulfate (free: $y = 0.21x + 6.4$; $R^2 = 1.00$; total: $y = 0.10x + 6.5$; $R^2 = 1.00$).

The difference in RR between free and total concentrations might imply that the equilibrium could not be formed during the course of the dialysis session. For the weakly-bound *p*CG, for example, around 90% of the total concentration is unbound and is thus easily removed by the dialyzer, resulting in a comparable total and free reduction ratio. The RR of the highly-bound *p*CS, on the other hand, is different for free and total concentrations, and within the four hours of dialysis, the RR of the total concentration cannot follow the RR of the free concentration (around 4%). The equilibrium between free and bound *p*CS is continuously disturbed because of the dynamic process of dialysis. Therefore, the equilibrium (as it was pre-dialysis) cannot be restored during the dialysis session and explains the observations in Figure 4-2 and Figure 4-3.

Besides well-known aspects such as molecular weight, steric configuration and charge, main determinants for the limited removal of protein-bound solutes during dialysis may be extended to: (i) only the free fraction can be removed and (ii) the bound fraction is released slowly. This fits with the multi-pass device observations, as described by Eloot *et al.*, where removal of

protein-bound solutes was limited to the first two hours of dialysis [13]. In a kinetic modelling study from our group [32] based on intradialytic concentrations, we found an inverse correlation between dialyzer clearance and the %PB. Furthermore, total distribution volumes and intercompartment clearances (except for *p*CG), which are representative for solute retardation inside the patients, were also inversely correlated with the %PB. Thus, the present findings are in full agreement with those in the kinetic analysis.

It can be stated that during dialysis, first the free fraction will be removed and will cause a disequilibrium with the bound fraction, as well as with concentrations in the extra vascular spaces. This results in a continuous release of the bound fraction, respectively inflow from the extravascular space. These kinetics of protein-bound solutes were already extensively studied by our group showing the multi-compartmental behavior with vascular and extravascular spaces [33].

Enhancing the filtration of proteins is cumbersome, as this would result in hypoproteinemia. However, strategies could be developed that change the strength of the physical bond between the toxin and its ligand, to increase the free (dialyzable) solute concentration.

Recent research suggested a novel approach to increase the free fraction of the protein-bound solutes phenyl acetic acid (PAA), indoxyl sulfate and *p*-cresyl sulfate during dialysis by infusing a hypertonic solution at the dialyzer inlet [20–22]. This increased the local ionic strength at the blood inlet of the dialyzer, resulting in an enhanced release of uremic toxin from its protein binding site, most pronounced for the middle bound PAA (%PB around 60 %). With this approach, the clearance during *in vitro* dialysis was relatively most beneficial for the highly-bound IS and *p*CS [20]. These *in vitro* results might be promising, but the absence of hemolysis due to hyperosmolarity needs to be further investigated *in vivo*.

4.2.5 Conclusion

In this study, we explored the protein binding of uremic toxins in patients with different stages of CKD and during a hemodialysis session. The observed change in protein binding in CKD patients with advanced CKD stages might be due to post-translational modifications of proteins, characteristic for CKD progression. The observed results in HD patients explain why protein-bound solutes are difficult to remove by dialysis: a combination of the fact that (i) only the free fraction can pass the filter and (ii) the equilibrium, as it was pre-dialysis, cannot be restored during the dialysis session, as it is continuously disturbed. This can be explained by the kinetics

of these protein-bound uremic toxins: once the free fraction is removed, the equilibrium with the bound fraction and the extra vascular space is disturbed, causing a potential release of the bound fraction, respectively inflow from the extravascular space [33].

4.2.6 Acknowledgments

The first author is supported by The Research Foundation – Flanders (FWO Vlaanderen, Project Number G0A4614N) and acknowledges Maria Van Landschoot and Marie-Anne Waterloos for the analysis of the blood samples.

4.2.7 Supplementary figures

Figure 4-5 Percentage protein binding (%PB) *versus* creatinine clearance of CKD patients for: (A) *p*-cresyl glucuronide; (B) hippuric acid; (C) indole-3-acetic acid; (D) indoxyl sulfate (with Spearman p- and R-value); and (E) *p*-cresyl sulfate.

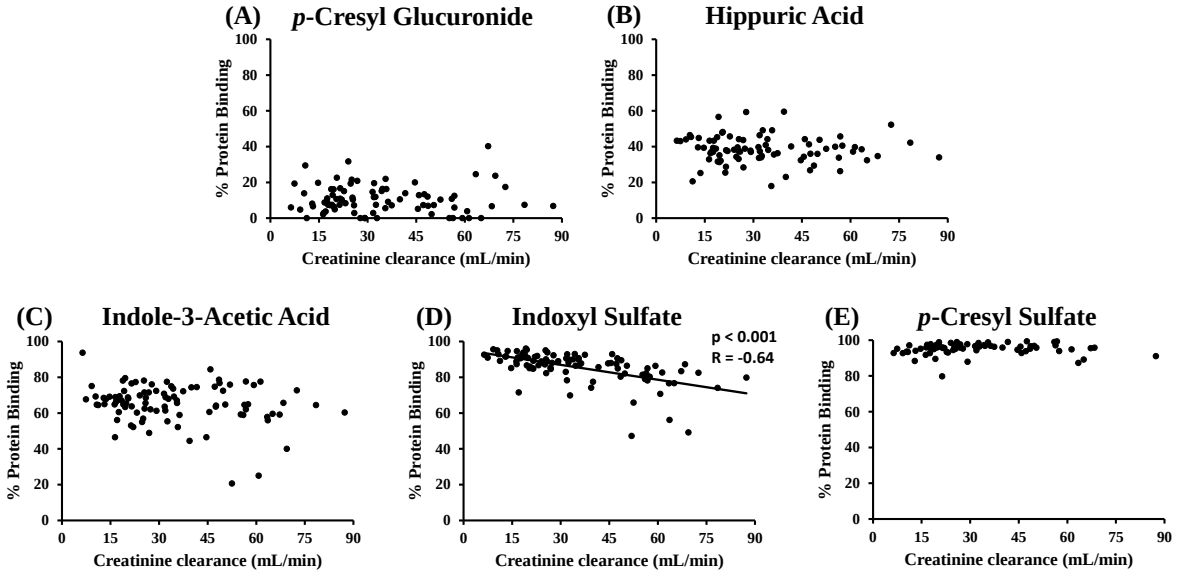


Figure 4-6 Percentage protein binding (%PB) *versus* albumin concentration of CKD patients for: (A) *p*-cresyl glucuronide; (B) hippuric acid; (C) indole-3-acetic acid; (D) indoxyl sulfate; and (E) *p*-cresyl sulfate.

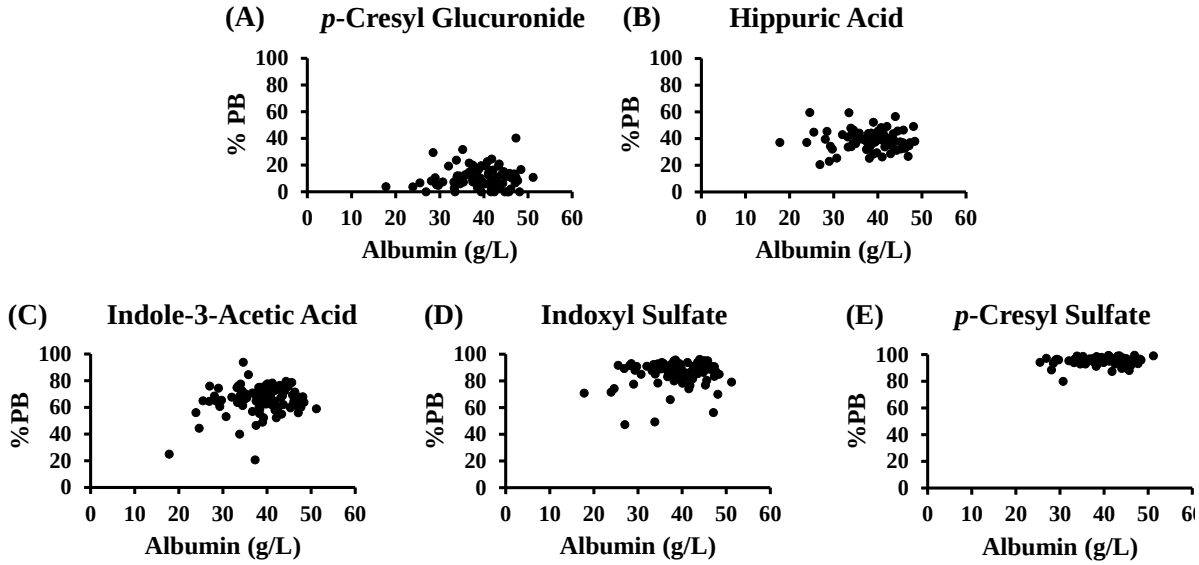
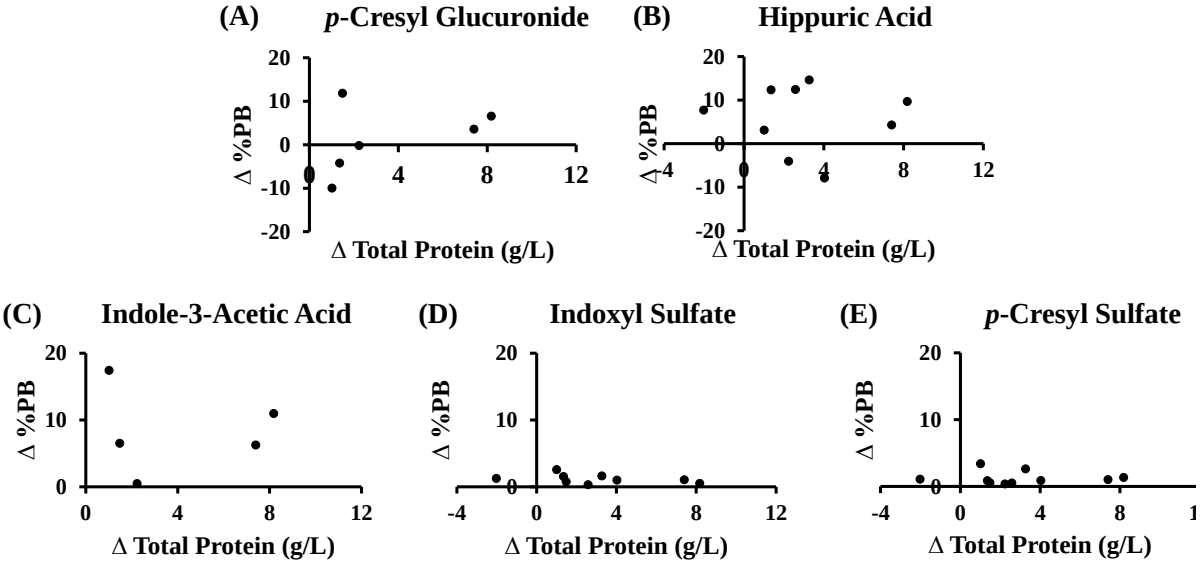


Figure 4-7 Change (Δ) in percentage protein binding (%PB) *versus* change in total protein concentration of HD patients at the inlet and the outlet of the dialyzer, after 120 min of dialysis for: (A) *p*-cresyl glucuronide; (B) hippuric acid; (C) indole-3-acetic acid; (D) indoxyl sulfate; and (E) *p*-cresyl sulfate.



4.3 References

1. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clarck W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jörres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W (2003) Review on uremic toxins: Classification, concentration and interindividual variability. Am J Soc Nephrol 63:1934–1943. doi: 10.1046/j.1523-1755.2003.00924.x

2. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A (2012) Normal and Pathologic Concentrations of Uremic Toxins. J Am Soc Nephrol 23:1258–1270. doi: 10.1681/ASN.2011121175

3. Schepers E, Meert N, Glorieux G, Goeman J, Van der Eycken J, Vanholder R (2007) P-cresylsulphate, the main in vivo metabolite of p-cresol, activates leucocyte free radical production. Nephrol Dial Transplant 22:592–596. doi: 10.1093/ndt/gfl584

4. Adijiang A, Goto S, Uramoto S, Nishijima F, Niwa T (2008) Indoxyl sulphate promotes aortic calcification with expression of osteoblast-specific proteins in hypertensive rats. Nephrol Dial Transplant 23:1892–901. doi: 10.1093/ndt/gfm861

5. Barreto FC, Barreto D V, Liabeuf S, Meert N, Glorieux G, Temmar M, Choukroun G, Vanholder R, Massy ZA (2009) Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. Clin J Am Soc Nephrol 4:1551–1558. doi: 10.2215/CJN.03980609

6. Meijers BKI, Van kerckhoven S, Verbeke K, Dehaen W, Vanrenterghem Y, Hoylaerts MF, Evenepoel P (2009) The Uremic Retention Solute p-Cresyl Sulfate and Markers of Endothelial Damage. Am J Kidney Dis 54:891–901. doi: 10.1053/j.ajkd.2009.04.022

7. Liabeuf S, Barreto D V., Barreto FC, Meert N, Glorieux G, Schepers E, Temmar M, Choukroun G, Vanholder R, Massy Z A. (2010) Free p-cresylsulphate is a predictor of mortality in patients at different stages of chronic kidney disease. Nephrol Dial Transplant 25:1183–1191. doi: 10.1093/ndt/gfp592

8. Jourde-Chiche N, Dou L, Cerini C, Dignat-George F, Brunet P (2011) Vascular Incompetence in Dialysis Patients-Protein-Bound Uremic Toxins and Endothelial Dysfunction. Semin Dial 24:327–337. doi: 10.1111/j.1525-139X.2011.00925.x

9. Sirich TL, Aronov PA, Plummer NS, Hostetter TH, Meyer TW (2013) Numerous protein-bound solutes are cleared by the kidney with high efficiency. Kidney Int 84:585–90. doi: 10.1038/ki.2013.154

10. Vanholder R, Schepers E, Pletinck A, Nagler E, Glorieux G (2014) The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review. J Am Soc Nephrol 1–11. doi: 10.1681/ASN.2013101062

11. Vanholder R, Pletinck A, Schepers E, Glorieux G (2018) Biochemical and Clinical Impact of Organic Uremic Retention Solutes: A Comprehensive Update. Toxins (Basel) 10:33. doi: 10.3390/toxins10010033

12. Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339–347. doi: 10.1053/ajkd.2002.34518

13. Eloot S, Van Biesen W, Axelsen M, Glorieux G, Pedersen RS, Heaf JG (2015) Protein-bound solute removal during extended multipass versus standard hemodialysis. BMC Nephrol 16:57. doi: 10.1186/s12882-015-0056-y

14. Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S (2015) Exploring Protein Binding of Uremic Toxins in Patients with Different Stages of Chronic Kidney Disease and during Hemodialysis. Toxins (Basel) 7:3933–3946. doi: 10.3390/toxins7103933

15. Sirich TL, Meyer TW, Gondouin B, Brunet P, Niwa T (2014) Protein-Bound Molecules: A Large Family With a Bad Character. Semin Nephrol 34:106–117. doi: 10.1016/j.semnephrol.2014.02.004

16. Meijers BKI, Bammens B, Verbeke K, Evenepoel P (2008) A Review of Albumin Binding in CKD. Am J Kidney Dis 51:839–850. doi: 10.1053/j.ajkd.2007.12.035

17. Gajjala PR, Fliser D, Speer T, Jankowski V, Jankowski J (2015) Emerging role of post-translational modifications in chronic kidney disease and cardiovascular disease. Nephrol Dial Transplant 1–11. doi: 10.1093/ndt/gfv048

18. de Loor H, Meijers BKI, Meyer TW, Bammens B, Verbeke K, Dehaen W, Evenepoel P (2009) Sodium octanoate to reverse indoxyl sulfate and p-cresyl sulfate albumin binding

in uremic and normal serum during sample preparation followed by fluorescence liquid chromatography. J Chromatogr A 1216:4684–4688. doi: 10.1016/j.chroma.2009.04.015

19. Viaene L, Annaert P, De Loor H, Poesen R, Evenepoel P, Meijers B (2013) Albumin is the main plasma binding protein for indoxyl sulfate and p-cresyl sulfate. Biopharm Drug Dispos 34:165–175. doi: 10.1002/bdd.1834

20. Böhringer F, Jankowski V, Gajjala PR, Zidek W, Jankowski J (2015) Release of Uremic Retention Solutes from Protein Binding by Hypertonic Predilution Hemodiafiltration. ASAIO J 61:55–60. doi: 10.1097/MAT.0000000000000166

21. Devine E, Krieter DH, Rüth M, Jankovski J, Lemke HD (2014) Binding affinity and capacity for the uremic toxin indoxyl sulfate. Toxins (Basel) 6:416–430. doi: 10.3390/toxins6020416

22. Krieter DH, Korner T, Devine E, Ruth M, Jankowski J, Wanner C, Lemke HD (2014) Pilot Trial on Ionic Strength Hemodiafiltration, A Novel Dialysis Technique for Increased Protein Bound Toxin Removal. Nephrol Dial Transplant 29:211–212.

23. Liabeuf S, Glorieux G, Lenglet A, Diouf M, Schepers E, Desjardins L, Choukroun G, Vanholder R, Massy ZA (2013) Does P-Cresylglucuronide Have the Same Impact on Mortality as Other Protein-Bound Uremic Toxins? PLoS One. doi: 10.1371/journal.pone.0067168

24. Cockcroft DW, Gault MH (1976) Prediction of creatinine clearance from serum creatinine. Nephron 16:31–41. doi: 10.1159/000180580

25. Eloot S, Van Biesen W, Glorieux G, Neirynck N, Dhondt A, Vanholder R (2013) Does the adequacy parameter kt/vurea reflect uremic toxin concentrations in hemodialysis patients? PLoS One. doi: 10.1371/journal.pone.0076838

26. Meert N, Schepers E, Glorieux G, Van Landschoot M, Goeman JL, Waterloos MA, Dhondt A, Van Der Eycken J, Vanholder R (2012) Novel method for simultaneous determination of p-cresylsulphate and p-cresylglucuronide: Clinical data and pathophysiological implications. Nephrol Dial Transplant 27:2388–2396. doi: 10.1093/ndt/gfr672

27. Kees MG, Wicha SG, Seefeld A, Kees F, Kloft C (2014) Unbound fraction of vancomycin in intensive care unit patients. J Clin Pharmacol 54:318–323. doi: 10.1002/jcph.175

28. Stove V, Coene L, Carlier M, Waele JJ De, Fiers T, Verstraete AG (2015) Measuring Unbound Versus Total Vancomycin Concentrations in Serum and Plasma : Methodological Issues and Relevance. Ther Drug Monit 37:180–187.

29. Rueth M, Lemke H-D, Preisinger C, Krieter D, Theelen W, Gajjala P, Devine E, Zidek W, Jankowski J, Jankowski V (2015) Guanidinylations of albumin decreased binding capacity of hydrophobic metabolites. Acta Physiol 215:13–23. doi: 10.1111/apha.12518

30. Perucca E (1980) Plasma Protein Binding of Phenytoin in Health and Disease: Relevance to Therapeutic Drug Monitoring. Ther Drug Monit 2:331–344.

31. Johannessen Landmark C, Johannessen SI, Tomson T (2012) Host factors affecting antiepileptic drug delivery-Pharmacokinetic variability. Adv Drug Deliv Rev 64:896–910. doi: 10.1016/j.addr.2011.10.003

32. Eloot S, Vanholder R (2013) How to Adapt Hemodialysis Strategies to Remove Protein-Bound Solutes More Adequately. Nephrol Dial Transplant 28:31–32.

33. Eloot S, Vanholder R (2012) Kinetics of protein-bound solutes during hemodialysis. Int J Artif Organs 35:583.

Chapter 5

Plasma protein binding of uremic toxins and antibiotics in healthy subjects *versus* hemodialysis patients

5.1 Introduction

It is often hypothesized that the percentage protein binding (%PB) of different protein-bound uremic toxins (PBUTs) is affected by a competition between them to bind to albumin [1–3]. Furthermore, it is suggested that binding sites on albumin might be saturated due to the accumulation of PBUTs in patients with CKD, resulting in lower percentages of protein binding [4]. Finally, many patients with CKD take medication of which the active drug compound may bind to plasma proteins as well. Consequently, these drugs may compete for the same binding site on albumin as an uremic toxin, influencing the %PB of the drug or toxin [1,5–12].

In this chapter, first the binding characteristics of a selected panel of PBUTs (*i.e.* HA, IAA, IS and *p*CS) is discussed as well as their related competition, in physiologic relevant matrices (*i.e.* serum). Second, the effect of CKD on the %PB of vancomycin and teicoplanin, *i.e.* two antibiotics which are commonly used for the treatment of infections in HD patients, as well as the %PB of the same panel of PBUTs is described in an *in vitro* setting.

5.2 Exploring binding characteristics and the related competition of different protein-bound uremic toxins

Based on: Deltombe O, de Loor H, Glorieux G, Dhondt A, Van Biesen W, Meijers B, Eloot S (2017) Exploring binding characteristics and the related competition of different protein-bound uremic toxins. Biochimie 139:20–26. doi: 10.1016/j.biochi.2017.05.010

5.2.1 Abstract

Little is known about potential differences in binding characteristics of protein-bound uremic toxins (PBUTs) in patients with chronic kidney disease (CKD) versus healthy controls. The question arises whether eventual differences are attributed to (i) the elevated levels of competing uremic toxins, and/or (ii) post-translational modifications of albumin.

We evaluated the binding characteristics of hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS), and *p*-cresyl sulfate (*p*CS) by deriving a binding curve in three distinct conditions: (i) serum from healthy controls (healthy serum), (ii) blank serum from hemodialysis patients (blank HD serum; *i.e.* cleared from uremic toxins), and (iii) non-treated serum from HD patients (HD serum). Additionally, the mutual binding competition of these uremic toxins was studied in blank HD in pairs. In both experiments, equilibrium dialysis (37 °C, 5 h) was used to separate the free and bound fractions of each PBUT. Free and total PBUT concentrations were quantified by an ultra-high performance liquid chromatography method with tandem mass spectrometer detection and the percentage protein binding (%PB) of each PBUT was calculated.

For all four compounds, the binding capacity of healthy serum was higher than blank HD serum, which was comparable to non-treated HD serum, except for HA. The competition experiments revealed that at high uremic concentrations, mutual competition was observed for the strongly bound PBUTs IS and *p*CS. The %PB of the weakly bound HA and IAA was lower (trend) only for the addition to blank HD serum containing the strongly bound IS or pCS.

There is an intrinsic impact on PB in uremia, revealing a lower binding capacity, as compared to healthy controls. Competitive binding is only relevant for the strongly bound PBUTs at high uremic concentrations. In addition, at least part of the effect on binding capacity may be attributed to post-translational modifications of albumin.

5.2.2 Introduction

Chronic kidney disease (CKD) is characterized by the retention of a large number of compounds that, under normal conditions, are excreted by the healthy kidneys. Numerous retention solutes have a negative effect on many biological functions and are therefore called uremic toxins [13–21]. Among them, the protein-bound uremic toxins (PBUTs) [22,23] mainly bind to albumin and this in different degrees [24,25]. The range in percentage protein binding (%PB) implies a differential removal of these toxins during hemodialysis, since only the free fraction can pass conventional dialyzer membranes [26].

Several studies reported a decreased %PB for some of these PBUTs in uremic serum or plasma, compared to normal serum or plasma [4,25–28]. In these studies, it was suggested that the decrease in %PB was attributed to either (i) the elevated PBUT concentrations in CKD and hemodialysis (HD) patients, resulting in a competition among the PBUTs to bind to albumin, or (ii) post-translational modifications (PTMs) of albumin in CKD and HD patients by e.g. oxidation [29–31], glycation [32–34], carbamylation [35–37], or guanidinylation [38] of lysine residues. However, to date, it is not clear whether the observed decrease in %PB of PBUTs in CKD and HD patients is attributed to (i) the competition between them, depending on their degree of binding (*i.e.* %PB) or binding affinity, (ii) the modification of albumin, or (iii) the combination of both.

In the past, a substantial number of binding studies were performed to unravel the binding characteristics of the studied PBUTs [1,25,28,39–44], but only few of them discussed their mutual competitive binding behavior in detail [25,41,43,44]. In most cases, the interaction between indoxyl sulfate (IS) and *p*-cresylsulfate (*p*CS) was discussed, whereas – to the best of our knowledge – none of these articles discussed the competition with other, less bound UTs such as hippuric acid (HA) or indole-3-acetic acid (IAA). Furthermore, most *in vitro* binding experiments were conducted in solutions prepared from commercially available human serum albumin powder (HSA) and did not take into account the PTMs affecting albumin as observed in CKD and HD patients [24,38,45], and their possible effect on the %PB of PBUTs [38].

In this work, we intended to gain more insight into the binding of HA, IAA, IS and *p*CS by evaluating their binding characteristics in three distinct conditions: (i) serum from healthy controls, (ii) serum from HD patients that was cleared from uremic toxins (*i.e.* blank HD serum) and (iii) non-treated serum from HD patients. Additionally, the mutual competitive binding of

these uremic toxins was studied by spiking blank HD serum with two PBUTs in pairs, having one PBUT in a varying concentration.

5.2.3 Materials and methods

5.2.3.1 Sample collection

Blood samples from twelve healthy controls and pre-dialysis blood samples from sixty-seven stable HD patients were collected in Venosafe serum tubes (Terumo Europe, Leuven, Belgium). After clotting, these tubes were centrifuged [Beckman Coulter X-15R centrifuge, 3000 rpm (range: 784 *g* – 2095 *g)*, 10 min] and aliquots were stored at -80 °C. After thawing, four different serum pools were prepared according to the requirements of the four experiments, *i.e.* the construction of a binding curve in (i) healthy serum, (ii) blank HD serum and (iii) non-treated HD serum and the competition experiments in (iv) blank HD serum. The blank HD sera were cleared from uremic toxins by a 24 h *in vitro* dialysis procedure, followed by the addition of activated charcoal, as recently described by de Loor *et al.* [46]. Each pool was stored at -80 °C until the experiments were performed.

This study was performed according to the Declarations of Helsinki, was approved by the local Ethical Committee (2010/033 for healthy serum, 2015/0571 for non-treated HD serum and 2015/0932 for blank HD serum), and all patients gave their written informed consent.

5.2.3.2 Chemicals

HA, IAA, IS and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA) and *p*CS from TCI Chemicals (Zwijndrecht, Belgium). Water (HPLC grade) was purchased from Acros Organics (Thermo Scientific, Geel, Belgium). All stock solutions were prepared in PBS buffer and stored at -20 °C.

5.2.3.3 Binding characteristics and mutual competition

Binding curves were constructed, in a single run, for the four PBUTs by spiking healthy serum, blank HD serum and non-treated HD serum samples (190 µL) with eight concentrations (10 µL, 20x stock solutions of the final concentration) of either HA, IAA, IS or *p*CS. For each compound, the theoretical final concentrations were 100, 250, 500, 750, 1000, 2500, 5000 and

10000 µmol/L, corresponding to normal physiological [23] over uremic [23] up to supra-physiological concentrations to ensure binding saturation. Finally, 150 µL of the spiked serum was used in equilibrium dialysis.

In a second series of experiments, the mutual binding competition of all four studied PBUTs was investigated by spiking blank HD serum (1980 µL) with a concentration (20 µL, 100x stock solution of the final concentration) of one PBUT to reach a final uremic concentration of 100 µmol/L for *p*CS and IS, 10 µmol/L for IAA and 400 µmol/L for HA [23], followed by an incubation step (37 °C, 30 min). Subsequently, the spiked blank HD serum was divided into aliquots of 190 µL, and the so-called competing PBUT was added to each aliquot in different concentrations (10 µL, 20x stock solutions of the final concentration), up to high uremic concentrations [23], followed by a second incubation step (37 °C, 30 min). Finally, 150 µL of the serum was used in equilibrium dialysis. These experiments were performed in triplicate.

5.2.3.4 Equilibrium Dialysis

In both series of experiments, equilibrium dialysis (ED) was used to separate the free fractions using a HTDialysis 96b system (HTDialysis, Connecticut, USA). The dialysis membranes, consisting of regenerated cellulose with a molecular weight cut-off of 3.5 kDa, were hydrated and used according to the manufacturer's guidelines. The spiked serum (150 µL) was dialyzed against a PBS solution (150 µL, pH = 7.4, ionic strength = 0.15 mol/L) for 5 hours at 37 °C on a reciprocating shaker and an adhesive film was used to seal the wells. After 5 hours, equilibrium was reached (determined in a pilot experiment, data not shown) and samples were taken from both sides of the dialysis chamber and stored at -80 °C until bulk analysis.

5.2.3.5 Analyses

Total and free PBUT concentrations were determined by an Acquity H Class (Waters, Zellik, Belgium) ultra-high performance liquid chromatography system. Chromatographic separation was performed on an Acquity CSH Fluoro Phenyl column (50 x 2.5 mm, 1.7 µm, Waters, Zellik, Belgium) with an Acquity CSH Fluoro Phenyl VanGuard pre-column (10 x 2.5 mm, 1.7 µm, Waters, Zellik, Belgium) and PBUTs were detected using a Xevo TQS tandem mass spectrometer (Waters, Zellik, Belgium). The sample preparation and chromatographic analysis method are described in more detail by de Loor *et al.* [46].

Total protein and albumin analyses were performed in the routine laboratory of Ghent University Hospital on a Cobas 8000 c701 (total protein) and c502 (albumin) analyzer (Roche Diagnostics, Mannheim, Germany) using the biuret and bromocresol green method, respectively.

5.2.3.6 Calculations

Total protein and albumin concentrations were measured in all serum pools before and after 5 hours of ED to check possible dilution effects caused by oncotic pressure. Therefore, 3 x 150 µL serum of each pool was dialyzed against 150 µL PBS buffer and 150 µL serum of the same pool. ED of serum against PBS resulted in a decrease in protein concentrations (*i.e.* 10 ± 0 % for healthy and HD serum and 6 ± 2 % for blank HD serum, all $p < 0.05$), whereas no change in protein concentration (*i.e.* 0 ± 2 %) was observed when dialyzing against serum of the same pool. Consequently, correction for these dilution effects was performed according to Banker *et al.*[47].

The non-covalent binding of PBUTs with proteins results in a complex (FP) which is assumed to be in a dynamic equilibrium with the free fraction of uremic toxins (F) and proteins (P):

$$FP \rightleftharpoons F+P \qquad \text{(Eq. 5-1)}$$

From this, a corresponding dissociation constant (K_d) can be defined as:

$$K_d = \frac{C_F\, C_P}{C_{FP}} \qquad \text{(Eq. 5-2)}$$

Where C_F is the free PBUT concentration, C_P is the protein concentration without bound ligands, and C_{FP} is the complex concentration. The dissociation constant represents the concentration of the free PBUT at which the binding sites on albumin are half occupied (*i.e.* $C_P = C_{FP}$). Consequently, the higher K_d, the weaker the affinity of the PBUT for albumin.

The %PB was calculated from the measured free (C_F) and total (C_T) PBUT concentrations:

$$\%PB = \left(1- \frac{C_F}{C_T}\right) \text{ x } 100\% \qquad \text{(Eq. 5-3)}$$

To correct for differences in albumin concentrations between the serum pools, the bound concentrations (*i.e.* $C_T - C_F$) were normalized to the measured albumin concentrations (C_{Alb}) and were expressed as the binding coefficient (B):

$$B = \frac{C_T - C_F}{C_{Alb}} \quad \text{(Eq. 5-4)}$$

SigmaPlot version 13 (Systat Software, San Jose, CA, USA) was used to fit the one site (Eq. 5-5) and two site (Eq. 5-6) binding models to the measured data B and C_F:

$$B = \frac{B_{max}\, C_F}{K_d + C_F} \quad \text{(Eq. 5-5)}$$

$$B = \frac{B_{max,1}\, C_F}{K_{d,1} + C_F} + \frac{B_{max,2}\, C_F}{K_{d,2} + C_F} \quad \text{(Eq. 5-6)}$$

Where B_{max} is the modeled maximal number of PBUT to a single albumin molecule (= binding capacity). Both models were compared with the standard error of the estimate and the Akaike Information Criterion (AIC) was calculated (*i.e.* the quality of each statistical model, relative to each of the other models). Models containing the lowest standard error and AIC value were retained. Binding curves were compared by the best-fit (B_{max}) values using a t-test. $P < 0.05$ was considered significant.

5.2.3.7 Statistics

SigmaPlot version 13 was used to perform statistical analyses. Data were checked for normality by a Shapiro-Wilk test. One-way repeated measures analysis of variance or Friedman tests with Tukey post hoc analysis were used as appropriate. $P < 0.05$ was considered significant.

5.2.4 Results

Table 5-1 presents the total protein and albumin concentrations, as well as the total PBUT concentrations of HA, IAA, IS and *p*CS as determined in each pool, used for the construction of binding curves and for the competition experiments. Total protein and albumin concentrations were the highest in the pool of healthy serum, followed by those measured in the pool of non-treated HD serum. The effectiveness of the applied clearing procedure is shown by the tremendous decrease in PBUT concentration (Table 5-1). Due to serum oncotic pressure, a small volume shift of dialysate was observed resulting in a small (12 – 20%) decrease in total protein and albumin concentration as well.

Table 5-1 Total protein, albumin, and total concentrations of hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) as determined in each pool, used for the construction of binding curves and in the competition experiments.

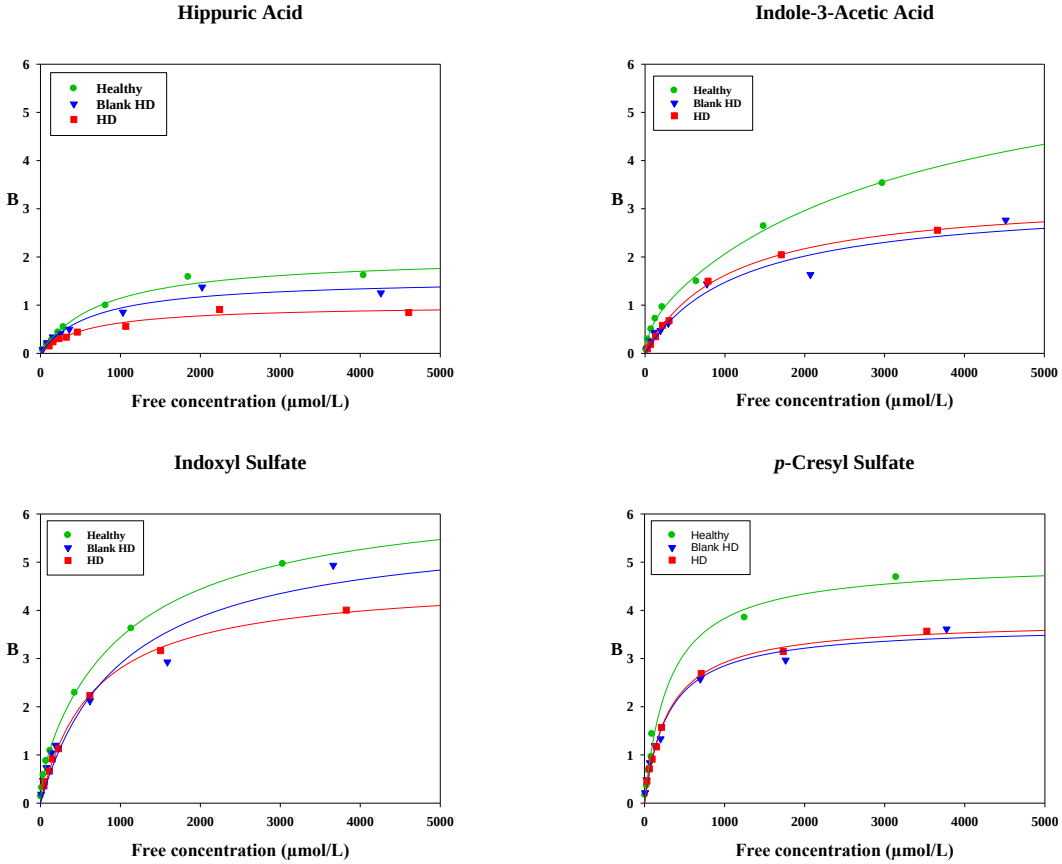
	Binding curves			Competition
	Healthy serum	**Blank HD serum**	**Non-treated HD serum**	**Blank HD serum**
Proteins (g/L)				
Total Protein	74.2	49.4	65.3	54.2
Albumin	48.1	29.4	40.1	33.6
Total PBUT (µmol/L)				
HA	5.00	< LOD[a]	190.07	< LOD[a]
IAA	2.59	0.18	8.26	0.40
IS	4.54	< LOD[b]	111.35	<LOQ[d]
*p*CS	11.63	< LOQ[c]	196.16	0.38

HD: hemodialysis, LOD: limit of detection, LOQ: limit of quantitation, [a]LOD (HA) = 0.5 µmol/L, [b]LOD (IS) = 0.03 µmol/L, [c]LOQ (*p*CS) = 0.3 µmol/L, [d]LOQ (IS) = 0.2 µmol/L.

The binding curves for HA, IAA, IS and *p*CS in healthy serum (green curve, dots), blank HD serum (blue curve, triangles), and non-treated HD serum (red curve, squares) are depicted in Figure 5-1 and the corresponding dissociation constant(s), K_d and binding capacity, B_{max} obtained after nonlinear regression are summarized in B: binding coefficient; $n = 1$.

Table 5-2. According to the standard error of the estimate and AIC comparison, a one site binding model was preferred for HA and *p*CS in healthy serum, whereas a two site binding model fitted the best for IAA and IS. The binding of the four studied PBUTs to albumin in blank HD serum and non-treated HD serum was best described by a one site binding model. The obtained K_d values for HA and *p*CS were in the same range (10^{-4}) for the different types of serum, whereas for IAA and IS, a high affinity (10^{-5}) site in healthy serum and a lower affinity site (10^{-4} – 10^{-3}) in blank HD serum and non-treated HD serum were found. For all four compounds, binding curves constructed in healthy serum reached higher B values than those in blank HD serum ($p < 0.05$ for HA, IAA, and *p*CS) and non-treated HD serum ($p < 0.05$ for HA, IS, and *p*CS; $p = 0.054$ for IAA). These findings are reflected in the B_{max} values, which were the lowest for HA (1.01 – 2.03), and where higher for IAA (3.20 – 6.05), IS (4.64 – 5.85), and *p*CS (3.69 – 5.00). This implicates that per albumin molecule, more ligands (*i.e.* PBUTs and other protein-bound solutes, such as drugs) can be bound in healthy serum than in blank HD and non-treated HD serum. Furthermore, the binding curve for HA in blank HD serum was higher than in non-treated HD serum ($p < 0.05$).

Figure 5-1 Binding curves for hippuric acid, indole-3-acetic acid, indoxyl sulfate and *p*-cresyl sulfate in healthy serum (green curve, dots), blank HD serum (blue curve, triangles), and non-treated HD serum (red curve, squares).



B: binding coefficient; n = 1.

Table 5-2 Dissociation constants, binding capacities, and AIC values as determined for hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) in healthy serum, blank HD serum, and non-treated HD serum.

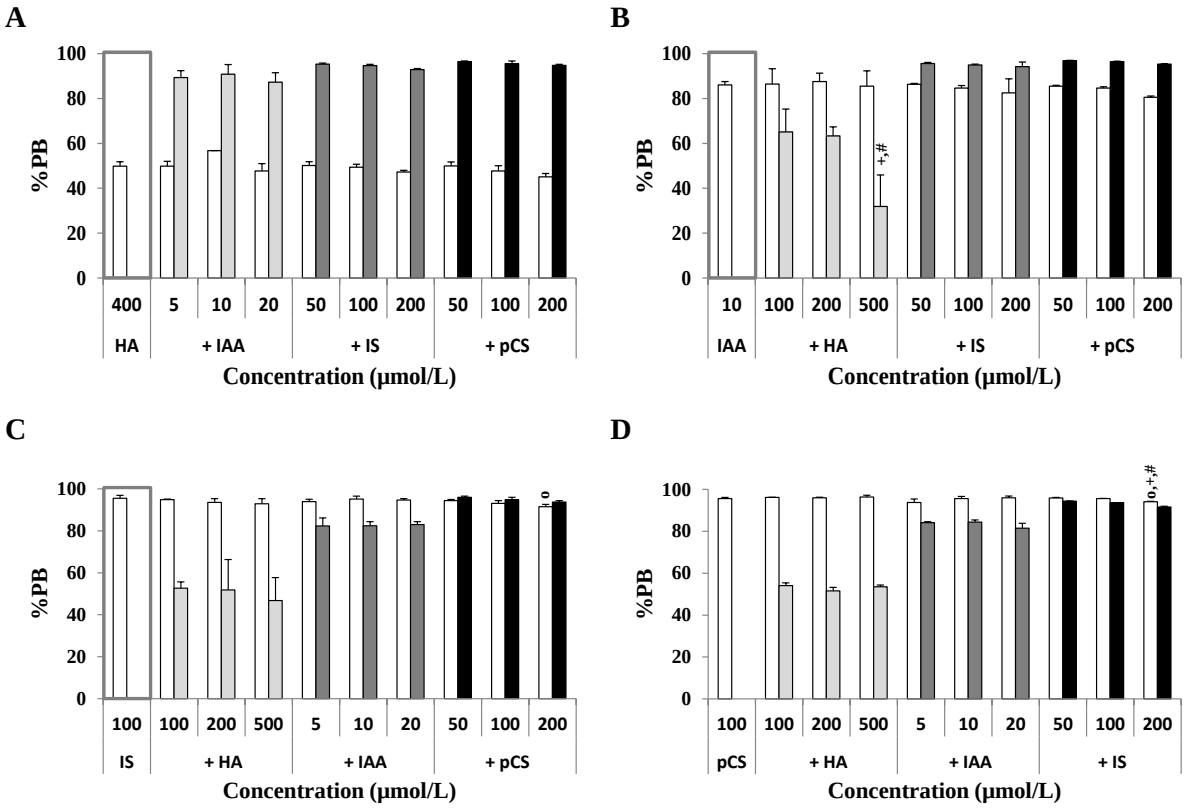
		Healthy serum	Blank HD	Non-treated HD serum
HA	K_d (mol/L)	$7.80\ 10^{-4}$	$6.63\ 10^{-4}$	$5.96\ 10^{-4}$
	B_{max}	2.03	1.56	1.01
	AIC	-31.4	-26.4	-34.4
IAA	K_{d1}/K_{d2} (mol/L)	$6.72\ 10^{-5}$ $3.27\ 10^{-3}$	$1.18\ 10^{-3}$	$1.04\ 10^{-3}$
	B_{max1}/B_{max2}	0.69 6.05	3.20	3.29
	AIC	-4.1	-14.7	-40.9
IS	K_{d1}/K_{d2} (mol/L)	$3.10\ 10^{-5}$ $1.11\ 10^{-3}$	$1.01\ 10^{-3}$	$6.60\ 10^{-4}$
	B_{max1}/B_{max2}	0.69 5.85	5.81	4.64
	AIC	-18.6	-5.1	-32.8
***p*CS**	K_d (mol/L)	$3.07\ 10^{-4}$	$2.94\ 10^{-4}$	$3.02\ 10^{-4}$
	B_{max}	5.00	3.69	3.80
	AIC	-13.6	-18.4	-30.0

HD: hemodialysis, K_d: dissociation constant, B_{max}: binding capacity, AIC: Akaike Information Criterion.

In the competition experiments, blank HD serum was first spiked with a fixed concentration of one PBUT (indicated by the grey frame at the left of each graph) and subsequently spiked with another PBUT (indicated by '+ PBUT' on the x-axis of each graph) in a stepwise varying

concentration and the obtained %PB of these PBUTs are shown in Figure 5-2 and Table 5-4 in 5.2.8 Supplementary table). In the serum samples spiked with either IS or *p*CS, the %PB of HA (panel A) or IAA (panel B) when spiked to final concentrations of 400 and 10 µmol/L, respectively, was not affected. A decreased %PB for HA was observed when HA (500 µmol/L) was added to serum containing 10 µmol/L IAA (panel B). Thus, the effect seemed to be rather small for the weakly bound HA and IAA. The addition of a maximum concentration of 200 µmol/L *p*CS and IS to serum containing 100 µmol/L IS (panel C) and *p*CS (panel D), respectively, was found to decrease the %PB of IS and *p*CS significantly, revealing a mutual competition between the strongly bound IS and *p*CS.

Figure 5-2 The percentage protein binding (%PB) of hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) as determined in blank HD serum spiked with one PBUT to reach a final uremic concentration (indicated by the grey frame at the left of each graph), followed by the addition of a competing PBUT, up to high uremic concentrations.



^{o} $p < 0.05$ *versus* no competitor added, $^{+}$ $p < 0.05$ *versus* the lowest concentration of competitor added, $^{\#}$ $p < 0.05$ *versus* the middle concentration of competitor added; $n = 3$.

A trend of lower %PB of HA was observed when added to 100 µmol/L *p*CS (51.6 – 54.1%) and IS (46.8 – 52.7%), as compared to serum containing 10 µmol/L IAA (for 100 and 200 µmol/L HA; 63.3 – 65.1%). A similar trend was observed for the addition of IAA to 100 µmol/L *p*CS (81.5 – 84.4%) or IS (82.3 – 83.0%), compared to serum containing

400 µmol/L HA (87.3 – 90.9%), demonstrating a possible influence of the type of PBUT that is present in the serum.

5.2.5 Discussion

In this work, the binding characteristics of HA, IAA, IS and *p*CS are described in healthy serum, blank HD serum, and non-treated HD serum. Furthermore, the mutual competitive binding of these four PBUTs was studied in pairs, using different concentrations of individual compounds. The main findings of this work are: (i) for all four compounds, the binding capacity of healthy serum was higher than blank HD serum, which was comparable to non-treated HD serum, except for HA and (ii) at high uremic concentrations, mutual competitive binding was observed between the strongly bound PBUTs IS and *p*CS. The %PB of the weakly bound HA and IAA was lower (trend) only for the addition to blank HD serum containing the strongly bound IS or *p*CS.

No differences in binding curves were observed for IAA, IS and *p*CS between blank HD and non-treated HD serum, despite the large amount of competing PBUTs in the latter serum pool. For HA, however, the binding curve constructed in blank HD serum was situated between the curves in healthy serum and non-treated HD serum. This blank HD serum was cleared from PBUTs and thus competition between the different PBUTs can in this setting be assumed as negligible. Thus, for HA, competition seemed to play a certain role in the binding to albumin in HD serum, but was only observed in the mutual competition experiments for the addition of 500 µmol/L HA to 10 µmol/L IAA, and was performed in blank HD serum. Possibly, protein-bound toxins, other than those studied in the present competition experiments, and protein-bound drugs might play a role as well in this competition for HA.

Several studies discussed the reduced binding capacity of albumin for PBUTs [4,24,26,42] and for drug compounds [6,10,48,49] in CKD patients. In some of these papers, the decreased binding capacity was attributed to the elevated concentrations of uremic toxins in CKD patients [4,24], while a growing number of studies describe the effect of PTMs of the albumin molecules on the protein binding of PBUTs in CKD patients [24,38,45]. Rueth *et al.* demonstrated the influence of guanidinylation on the binding of IS to albumin and found that B_{max} values were significantly decreased in patients with CKD [38].

In

Table 5-3, an overview of binding characteristics for HA, IAA, IS, and *p*CS as well as the corresponding experimental conditions to obtain these results is presented, as found in the literature. Most studies reported that HA, IAA, IS, and *p*CS bind to albumin according to a two-site model, while we only found this for IAA and IS in healthy serum. Additionally, the binding capacity of albumin was previously reported to be much lower than the B_{max} values presented in this work. However, in the previous studies, the binding characteristics of HA, IAA, IS, and *p*CS were mostly evaluated without reaching saturation of the binding sites of albumin [25,28] and in most cases, the experiments were performed in a solution prepared from commercially available human serum albumin powder [1,3,38,39,41,42,50,51]. In our study, we aimed to reflect the physiologic and uremic situation the best by using healthy and HD serum, respectively. Moreover, a better estimate of the binding capacity of albumin could be obtained in these two groups by (almost) reaching binding saturation.

To the best of our knowledge, only the mutual competitive binding of IS and *p*CS was reported previously [42,43] and our findings are in line with those discussed by Meijers *et al.* as they showed that the mutual competition of IS and *p*CS in serum from both healthy controls and HD patients is small, but significant [43]. Watanabe *et al.* demonstrated a larger competitive effect when studying concentrations far beyond the uremic range [42].

Although we studied binding characteristics and binding competition in the most relevant matrix (*i.e.* serum), some issues need to be stressed. Firstly, in each experiment, the free fraction was obtained by equilibrium dialysis. In this method, it is known that samples are susceptible to pH shifts when they are in contact with the surrounding air [52]. Therefore, we used an adhesive film to close the system, minimizing contact with the air, and serum samples were dialyzed against a PBS buffer with pH = 7.4. Nevertheless, if any small shift in pH would have occurred, this would not have changed the %PB of the investigated PBUTs significantly, as we recently demonstrated [53]. Secondly, blank HD serum showed a slight decrease in protein concentration during the dialysis step, most likely caused by oncotic pressure. This small disadvantage does not outweigh the use of in vivo relevant modified human albumin present in blank HD serum. Furthermore, the presented binding curves and their corresponding characteristics were corrected for albumin concentration, and in the competition experiments, all tests were performed in the same pool of blank HD serum.

Table 5-3 Summary of dissociation constants, binding capacities, and experimental conditions found in literature, as determined for hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS).

	$K_{d,1}$	$K_{d,2}$	$B_{max,1}$	$B_{max,2}$	Matrix (µmol/L)	$C_{max,PBUT}$ (µmol/L)	Buffer	T (°C)	Method	Ref
HA	$6.49\ 10^{-5}$	$7.75\ 10^{-4}$	n.m.		25 (HSA)	1928	20 mM SPB	37	ITC	[51]
	$7.63\ 10^{-4}$		0.97		5 (HSA)	10	20 mM SPB	37	FQ	
	$1.00\ 10^{-4}$	$3.33\ 10^{-3}$	1.0	7.0	200 (HSA)	140	67 mM SPB	25	UF/ED	[50]
	$4.00\ 10^{-5}$	$1.50\ 10^{-3}$	9.2	23	336 (BSA)	300	100 mM SPB	25	POT	[39]
IAA	$4.76\ 10^{-6}$	$1.25\ 10^{-3}$	1.0	5.0	40 (HSA)	18	67 mM SPB	25	UF/ED	[50]
	$1.16\ 10^{-2}$		n.m.		360 (HSA)		67 mM P/SPB	37	ED	[1]
	$4.30\ 10^{-4}$	$8.70\ 10^{-3}$	1.2	2.9	336 (BSA)	300	100 mM SPB	25	POT	[39]
IS	$6.21\ 10^{-7}$	$1.20\ 10^{-4}$	1.0	3.0	40 (HSA)	24	67 mM SPB	25	UF/ED	[50]
	$8.20\ 10^{-1}$		0.97		900 (HSA)	30000	25 mM MES	37	ITC	[3]
	$3.20\ 10^{-4}$	$4.00\ 10^{-3}$	5.0	6.8	336 (BSA)	300	100 mM SPB	25	POT	[39]
	$1.02\ 10^{-5}$	$8.33\ 10^{-4}$	1.0	1.6	500 (HSA)	n.m.	PBS	25	UF	[42]
	$5.04\ 10^{-6}$	n.m.	0.72		547 (HSA)		PBS			
	$1.54\ 10^{-5}$	n.m.	0.74		Healthy plasma	1200	n.a.	37	ED/UF	[25]
	$2.51\ 10^{-5}$	n.m.	0.65		HD plasma					
	$1.34\ 10^{-5}$		0.54		Healthy plasma	150	n.a.	RT	UF	[28]
	$6.10\ 10^{-6}$		0.72		37.5 (Isolated healthy HSA)	150	PBS	RT	UF	[38]
	$2.71\ 10^{-6}$		0.23		37.5 (Isolated CKD HSA)					
***p*CS**	$4.29\ 10^{-3}$		1.02		900 (HSA)		10 mM SPB	37	ITC	[41]
	$1.00\ 10^{-5}$	$1.96\ 10^{-4}$	1.0	1.2	500 (HSA)	n.m.	PBS	25	UF	[42]
	$9.52\ 10^{-6}$	n.m.	1.05		547 (HSA)		PBS			
	$1.05\ 10^{-5}$	n.m.	0.78		Healthy plasma	1200	n.a.	37	ED/UF	[25]
	$2.83\ 10^{-5}$	n.m.	1.07		HD plasma					

K_d: dissociation constant in mol/L, B_{max}: binding capacity, HSA: human serum albumin solution, BSA: bovine serum albumin solution, $C_{max,PBUT}$: highest concentration of protein-bound uremic toxin, buffer: used to dissolve BSA or HSA, (P/)SPB: (potassium/) sodium phosphate buffer, PBS: phosphate buffered saline, MES: 2-(N-morpholino)ethanesulfonic acid, RT: room temperature, ITC: isothermal titration calorimetry, FQ: fluorescence quenching, UF: ultrafiltration, ED: equilibrium dialysis, POT: potentiometry, n.m.: not mentioned, n.a.: not applicable.

5.2.6 Conclusion

Using serum from healthy and HD patients, we adequately described the intrinsic impact on protein binding in uremia, revealing a lower binding capacity, as compared to healthy controls. Mutual competition was mainly observed for the strongly bound PBUTs and/or with high uremic concentrations. In addition, at least part of the effect on binding capacity may be attributed to PTMs of albumin in uremic patients. In the same line of the present study, it would be very interesting to investigate the possible competition of protein-bound drug compounds versus PBUTs in CKD and HD patients.

5.2.7 Acknowledgements

This project was supported by The Research Foundation – Flanders (FWO Vlaanderen, Project Number G0A4614N).

5.2.8 Supplementary table

Table 5-4 The percentage protein binding (%PB) of hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) as determined in blank HD serum spiked with a concentration of one PBUT (indicated by the black frame at the left of each panel) to reach a final uremic concentration, followed by the addition of a competing PBUT, up to high uremic concentrations.

A	C (µmol/L)	%PB competitor	%PB HA
HA	400	/	49.9 ± 1.9
+ IAA	5	89.3 ± 2.9	49.8 ± 2.2
	10	90.9 ± 14.5	56.8 ± 0.0
	20	87.3 ± 10.9	47.7 ± 3.3
+ IS	50	95.3 ± 3.8	50.1 ± 1.7
	100	94.7 ± 1.9	49.4 ± 1.2
	200	92.9 ± 1.5	47.2 ± 0.8
+ *p*CS	50	96.5 ± 0.6	49.9 ± 1.8
	100	95.5 ± 1.1	47.7 ± 2.3
	200	94.8 ± 0.7	45.1 ± 1.5

B	C (µmol/L)	%PB competitor	%PB IAA
IAA	10	/	86.1 ± 1.4
+ HA	100	65.1 ± 10.2	86.4 ± 6.8
	200	63.3 ± 4.0	87.6 ± 3.7
	500	$31.9^{+,\#}$ ± 14.0	85.6 ± 6.8
+ IS	50	95.7 ± 0.4	86.4 ± 0.4
	100	94.9 ± 0.4	84.7 ± 1.1
	200	94.3 ± 2.0	82.5 ± 6.2
+ *p*CS	50	96.9 ± 0.2	85.5 ± 0.3
	100	96.5 ± 0.2	84.7 ± 0.5
	200	95.4 ± 0.2	80.5 ± 0.6

C	C (µmol/L)	%PB competitor	%PB IS
IS	100	/	95.6 ± 1.4
+ HA	100	52.7 ± 2.9	94.9 ± 0.3
	200	51.8 ± 1.7	93.6 ± 1.8
	500	46.8 ± 0.9	92.9 ± 2.4
+ IAA	5	82.3 ± 0.4	94.0 ± 1.1
	10	82.5 ± 1.0	95.2 ± 1.3
	20	83.0 ± 2.3	94.6 ± 0.7
+ *p*CS	50	96.0 ± 0.1	94.4 ± 0.6
	100	94.9 ± 0.1	93.1 ± 1.3
	200	93.7 ± 0.3	91.5^{o} ± 1.1

D	C (µmol/L)	%PB competitor	%PB *p*CS
*p*CS	100	/	95.6 ± 0.6
+ HA	100	54.1 ± 1.3	96.2 ± 0.1
	200	51.6 ± 1.7	96.0 ± 0.2
	500	53.5 ± 0.9	96.4 ± 0.8
+ IAA	5	84.2 ± 0.4	93.7 ± 1.7
	10	84.4 ± 1.0	95.6 ± 1.0
	20	81.5 ± 2.3	96.1 ± 0.8
+ IS	50	94.4 ± 0.1	96.0 ± 0.2
	100	93.6 ± 0.1	95.6 ± 0.2
	200	91.7 ± 0.3	$94.2^{o,+,\#}$ ± 0.2

^{o} $p < 0.05$ *versus* no competitor added, $^{+}$ $p < 0.05$ *versus* the lowest concentration of competitor added,
$^{\#}$ $p < 0.05$ *versus* the middle concentration of competitor added. Values are expressed as mean ± standard deviation.

5.3 Protein binding of vancomycin and teicoplanin and the related competition with protein-bound uremic toxins in plasma from healthy subjects and patients on hemodialysis: an *in vitro* study

In preparation: Deltombe O, Stove V, Glorieux G and Eloot S, Protein binding of vancomycin and teicoplanin and the related competition with protein-bound uremic toxins in plasma from healthy subjects and patients on hemodialysis: an *in vitro* study.

5.3.1 Abstract

It is often hypothesized that the protein binding of drugs might be changed in patients with chronic kidney disease (CKD) as compared to patients with normal renal function, as a result of binding competition with protein-bound uremic toxins (PBUTs).

In the present study, the percentage protein binding (%PB) of two commonly administered antibiotics, *i.e.* vancomycin and teicoplanin, was studied *in vitro* by spiking a plasma pool from healthy subjects (only high) and from patients on hemodialysis (HD; low, medium and high) at different concentrations [22.5 (low), 45 (medium) and 90 (high) mg/L for vancomycin and 35 (low), 70 (medium) and 140 (high) mg/L for teicoplanin]. Our aims were (i) to find out whether the %PB of the antibiotics is affected in patients treated with HD and (ii) to evaluate the potential protein binding competition with the following PBUTs: hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS).

No difference in %PB for vancomycin (%PB = 58.9 % *versus* 60.0 %), nor for teicoplanin (%PB = 94.7 % *versus* 94.6 %) was observed after spiking healthy and HD plasma with a high concentration. But, a significant increase in %PB of vancomycin was found from low over medium to high concentration. When the protein binding of PBUTs in the presence of vancomycin was studied, a minor but significant increase of %PB was found for all four PBUTs, high drug concentration *versus* low, while a similar minor increase was present for high concentration *versus* no drug for IS and *p*CS. For medium concentration *versus* reference there was no difference. When the highly-bound teicoplanin was added to both plasma pools, no relevant changes in %PB of the studied PBUTs were observed.

Overall, it was concluded that (i) the %PB of vancomycin and teicoplanin was respectively slightly or not affected in plasma from HD patients and (ii) there were no relevant biological changes in the %PB of PBUTs, for the studied clinical concentrations, suggesting the absence of protein binding competition between the antibiotics *versus* the studied PBUTs.

5.3.2 Introduction

It is often hypothesized that the protein binding of drug compounds might be changed in patients with chronic kidney disease (CKD) as compared to patients with normal renal function [6, 9]. Therefore, the effect of CKD has previously been studied both *in vitro* and *in vivo* for a number of drugs. In general, the percentage protein binding (%PB) was found to be increased for basic drugs (e.g. clonidine (pK_a = 8.2) and propranolol (pK_a = 9.7) [6]) and decreased for acidic drugs (e.g. methotrexate (pK_a = 3.4) [6], valproic acid (pK_a = 5.1) [10, 48] and furosemide (pK_a = 4.25) [8, 54]). Neutral drug compounds were affected in a varying way resulting in either an increase (e.g. cimetidine (pK_a = 13[1] and 6.9[2]) [6]) or decrease (e.g. phenytoin (pK_a = 9.5[1] and -9[2]) [6, 48], theophylline (pK_a = 7.8[1] and -0.8[2]) [6], diazepam (pK_a = 2.9[2]) [6, 55]) in %PB. Additionally, for some other neutral (e.g. propofol (pK_a = 11.1[1] and -5[2]) [56]) and basic drugs (e.g. penbutolol (pK_a = 9.8) [57] and gentamicin (pK_a = 12.5) [58]), the %PB was not found to be changed in patients with CKD as compared to patients with normal renal function.

Decreased drug protein binding in patients with CKD is sometimes attributed to the binding competition with accumulated endogenous compounds [9], also called uremic retention solutes, or uremic toxins when they exert toxicity [22, 23]. Not only a large fraction of drug compounds, but also protein-bound uremic toxins (PBUTs), such as hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS), predominantly bind to albumin [25, 44]. Therefore, drugs and PBUTs may compete when they bind to the same binding site on albumin [9]. However, to the best of our knowledge, data supporting this hypothesis are only available for furosemide [8, 54]. A second cause of altered protein binding might be attributed to post-translational modifications (PTMs, e.g. oxidation [29–31], glycation [32–34], carbamylation [35–37] and guanidinylation [38]) of albumin in patients with CKD. These PTMs may result in a conformational change of the binding sites, resulting in a lower or higher binding affinity.

Vancomycin and teicoplanin are two protein-bound glycopeptide antibiotics which have a similar mechanism of action and can be used to treat infections caused by Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* [59–62]. While there is a high variability in the reported %PB for vancomycin, *i.e.* from 5 to 90 % [49, 63–69], the variability in the reported %PB values for teicoplanin is less, *i.e.* ranging from 70 to 99% [65, 70–72]. Both antibiotics are predominantly eliminated by renal excretion [70, 73]. Therefore, dosage adjustment is required in patients with CKD [60, 70, 74, 75]. Information on their free fraction,

[1]pKa for the acid and [2]pKa for the base

and thus %PB, in patients with CKD is scarce and has been described for vancomycin in only two papers [49, 63]. For teicoplanin, to the best of our knowledge, no protein binding studies in a CKD population were performed so far. However, knowledge on the free fraction might be important since only the free drug can travel through cell membranes towards the site of action to exert its pharmacological effect. Therefore, changes in protein binding are especially important when the %PB of a compound is high, such as for teicoplanin. In addition, no data are available on the possible protein binding competition of vancomycin and teicoplanin with PBUTs.

In this *in vitro* study, the %PB of vancomycin, teicoplanin as well as of a selected panel of PBUTs (*i.e.* HA, IAA, IS and *p*CS) was compared before (PBUTs only) and after spiking a pool of plasma, obtained from healthy subjects and from patients on hemodialysis (HD), with either vancomycin or teicoplanin.

5.3.3 Materials and methods

5.3.3.1 Sample collection

Blood samples from 13 healthy subjects and pre-dialysis blood samples from 16 stable HD patients were collected in Vacutainer K_2EDTA tubes (Becton Dickinson Company, New Jersey, USA). Subsequently, blood was centrifuged for 10 min at 2095 *g* at room temperature (RT) (Beckman Coulter X-15R centrifuge - VWR, Leuven, Belgium) and the obtained plasma was stored at -80 °C. After thawing, two different plasma pools were prepared, *i.e.* one of healthy plasma and one of HD plasma, and aliquots of 975 µL were stored at -80 °C until the experiments were started.

This study was conducted according to the Declarations of Helsinki, was approved by the Ethical Committee of Ghent University Hospital (2017/0162 and 2008/232) and all participants gave their written informed consent.

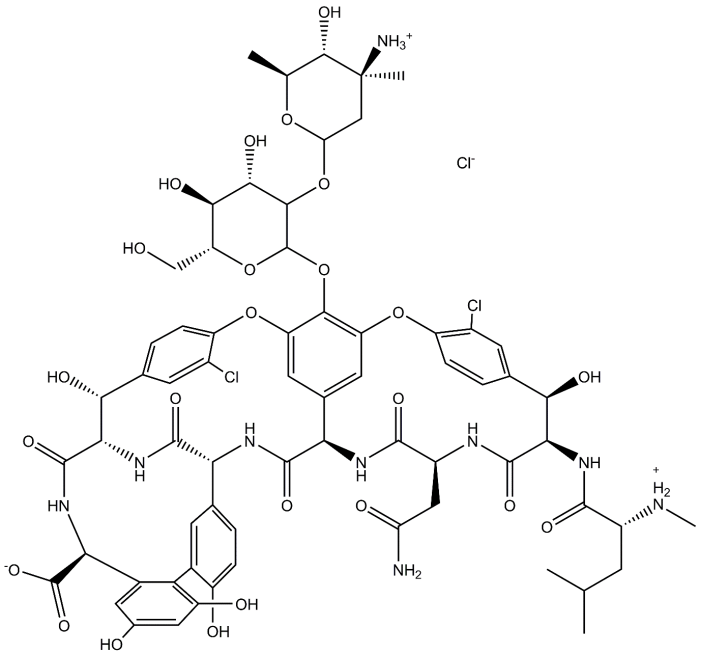
5.3.3.2 Chemicals

Vancomycin hydrochloride (500 mg) was purchased from Mylan (Hoeilaart, Belgium), teicoplanin (400 mg) from Sanofi-Aventis (Machelen, Belgium) and phosphate buffered saline (PBS) buffer pH = 7.4 from Invitrogen (Thermo Fisher Scientific, Ghent, Belgium).

5.3.3.3 Stock solutions

Vancomycin (500 mg, Figure 5-3) and teicoplanin (400 mg, Figure 5-4) were separately dissolved in 10 mL PBS to reach final theoretical concentrations of 50 g/L and 40 g/L, respectively. Subsequently, these solutions were further diluted to obtain a high, medium and low 40x stock solution of the final concentration. Theoretical concentrations of these 40x stock solutions were 3600 (high), 1800 (medium) and 900 (low) mg/L for vancomycin and 5600 (high), 2800 (medium) and 1400 (low) mg/L for teicoplanin, respectively.

Figure 5-3 Chemical structure of vancomycin hydrochloride.

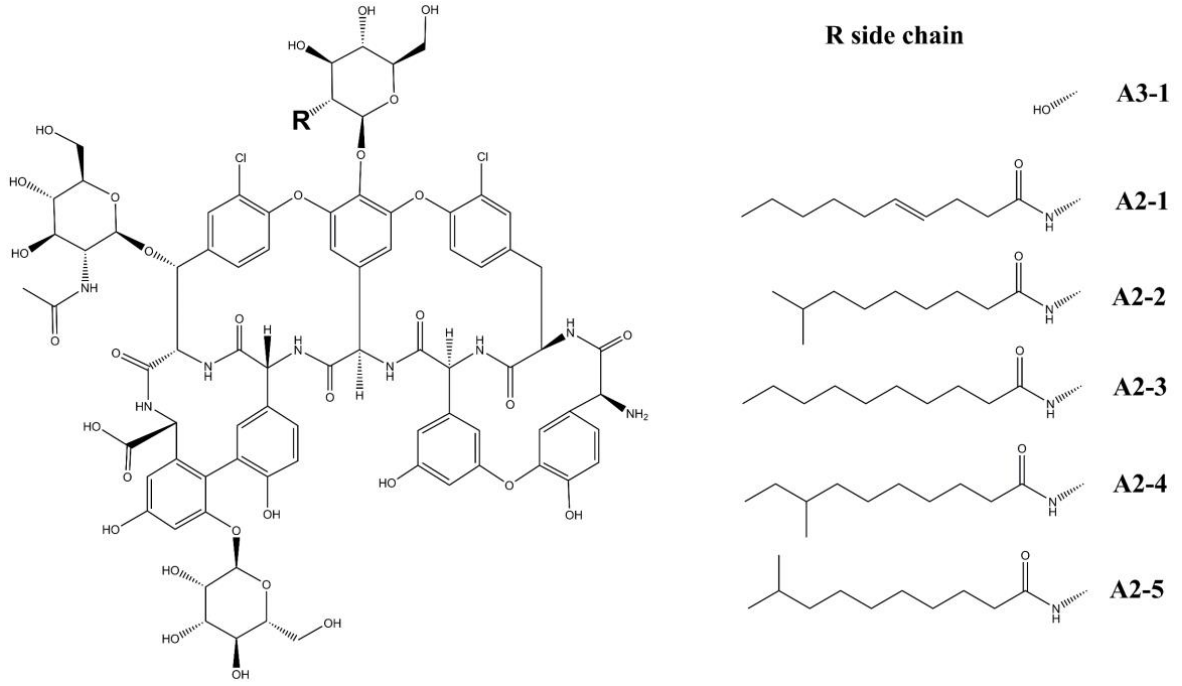


5.3.3.4 Vancomycin and teicoplanin spiking experiments

Aliquots (975 µL) of healthy and HD plasma pools were thawed and mixed. Subsequently, 25 µL of the 40x stock solution containing either a high vancomycin or teicoplanin concentration was added to healthy plasma (each n = 6) and 25 µL of the 40x stock solution containing either vancomycin or teicoplanin in high, medium or low concentration was added to HD plasma (each n = 6). In addition, reference samples (each n = 6) were included to study the baseline %PB of PBUTs in both plasma pools. Hereto, 25 µL PBS containing neither vancomycin nor teicoplanin was added to the reference samples. After spiking, all samples were mixed and incubated at RT for 30 min. Next, 500 µL plasma was transferred into Centrifree Ultrafiltration filters (Merck, Darmstadt, Germany), which were spun at 1885 g for 30 min at

37 °C as previously described for vancomycin [76]. Finally, ultrafiltrate (containing free PBUT and either free vancomycin or teicoplanin) and corresponding plasma samples (for total PBUT and either total vancomycin or teicoplanin quantification) were kept at 4 °C (max 24h) until sample preparation or were analyzed directly (vancomycin assay only).

Figure 5-4 Chemical structure of teicoplanin, which is composed of compounds A3-1, A2-1, A2-2, A2-3, A2-4 and A2-5.



5.3.3.5 Analyses

PBUTs. Plasma and ultrafiltrate samples (100 µL) were diluted by adding 260 µL HPLC grade water, heated for protein denaturation (30 min, 95 °C), cooled down on ice (10 min) and spun at 15588 *g* (10 min, RT, Beckman Coulter F241.5P Microfuge 18 – Analis, Ghent, Belgium). Subsequently, denaturated plasma samples were ultrafiltered to have all steps equal for the analysis of both free and total PBUTs. Next, 225 µL of each sample was transferred into an autosampler vial, 25 µL internal standard (fluorescein, 50 mg/L) was added and each sample was vortexed. Finally, all samples were placed in the autosampler at 4 °C and 18 µL was injected onto the column [53]. Total and free concentrations of hippuric acid (HA), indole-3 acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) were determined by an ultra-high performance liquid chromatography (UHPLC) instrument with ultraviolet (HA) and fluorescence (IAA, IS and *p*CS) detection, as previously described in more detail [53]. The within-run and between-run precision for both total and free PBUTs were below 2.0 % and

3.3 % for HA, 2.6 % and 6.3 % for IAA, 2.6 % and 4.8 % for IS, and 2.5 % and 6.0 % for *p*CS, respectively.

Vancomycin. Total and free vancomycin concentrations were assayed by a chemiluminescent microparticle immunoassay [76]. Plasma and ultrafiltrate samples were analyzed directly since no sample preparation was required for this assay. The within-run precision was < 6.4 % for both total and free vancomycin analyses [76].

Teicoplanin. In this method, 50 µL internal standard (trimipramine-D_3) was added to 50 µL plasma and ultrafiltrate samples. Subsequently, 200 µL of a MeOH/ACN (1:1, both LC-MS grade) solution was added and samples were vortexed and cooled down at -20 °C (30 min). Next, all samples were mixed using a Thermomixer (5 min, 1400 rpm, 10 °C – Eppendorf, Hamburg, Germany) and centrifuged (5 min, 16162 *g*, RT – Beckman Coulter Microfuge 16, VWR, Leuven, Belgium). Finally, 100 µL supernatant of each sample was transferred into an autosampler vial and was placed in the autosampler at 10 °C and 1 µL (total teicoplanin) or 2 µL (free teicoplanin) was injected onto the column. Total and free teicoplanin (A2-1 to A2-5 and A3-1 compounds) concentrations were determined by an UHPLC – high resolution mass spectrometry method (*in house* developed and validated method). The within-run and between-run precision for both total and free teicoplanin were 12.6 % and 11.5 %, respectively.

Total protein and albumin analyses were performed on a Cobas 8000 c701 (total protein) and c502 (albumin) analyzer (Roche Diagnostics, Mannheim, Germany) using the biuret and bromocresol green method, respectively.

5.3.3.6 Calculations

The %PB was calculated from the measured free (C_F) and total (C_T) concentration:

$$\%PB = \left(1 - \frac{C_F}{C_T}\right) \text{ x } 100\% \qquad \text{(Eq. 5-7)}$$

Statistical evaluation was performed with GraphPad Prism 4.00 for Windows (GraphPad Software, La Jolla, California USA). Data were checked for normality by a Shapiro-Wilk test. Most data are not normally distributed and are therefore expressed as median [25^{th} percentile (pct); 75^{th} pct]. Non-parametric tests, including Mann Whitney and Wilcoxon signed-rank test and Friedman tests with Dunn's post hoc analysis, were used when appropriate. $P < 0.05$ was considered significant.

5.3.4 Results

Total protein concentrations in the pool of healthy and HD plasma were 72.7 and 66.5 g/L, whereas albumin concentrations were 45.9 and 38.2 g/L, respectively.

5.3.4.1 Vancomycin

Data of the vancomycin spiking experiment are provided in Table 5-5. Addition of vancomycin to the HD plasma resulted in a median total vancomycin concentration of 21.9 (low); 41.8 (medium) and 88.3 (high) mg/L and median corresponding free concentrations of 10.7 (low); 19.3 (medium) and 35.6 (high) mg/L. No difference in %PB of vancomycin was found when healthy plasma was spiked with the highest concentration (%PB = 58.9 %) as compared to HD plasma (%PB = 60.0 %). In HD plasma, however, the median %PB of vancomycin increased significantly from 50.4 to 54.7 and 60.0 % when spiked with a low and median *versus* a high concentration, respectively.

Total PBUT concentrations of HA, IAA, IS and *p*CS as determined in the pool of healthy plasma and in the pool of HD plasma are also summarized in Table 5-5. After spiking of the healthy plasma pool with a high concentration of vancomycin, the total concentration of HA was increased (+17.4 %) with a decrease (-19.4 %) in free concentration, resulting in an increase in %PB (+33.7 %). Spiking HD plasma with increasing concentrations of vancomycin resulted in a significant decrease in free HA (-7.1 %), IAA (-10.9 %), IS (-18.1 %) and *p*CS (-14.2 %) concentrations when comparing the high concentration with the low concentration of vancomycin. Furthermore, a decrease in free IS (-14.3 %) and *p*CS (-10.6 %) concentration was also significant as compared to the reference samples. These changes in free PBUT concentration resulted in a significant increase in %PB, which was +9.4 % for HA and +4.8 % for IAA as compared to samples spiked with a low concentration and +0.9 % and +1.1 % for IS and +0.6 % and +0.9 % for *p*CS as compared to the %PB in the reference samples and samples spiked with a low concentration of vancomycin, respectively (Table 5-5).

5.3.4.2 Teicoplanin

Data of the teicoplanin experiment are summarized in Table 5-6. Addition of teicoplanin to the HD plasma resulted in a median total teicoplanin concentration of 39.5 (low); 86.2 (medium) and 145.8 (high) mg/L and median free teicoplanin concentrations of 2.2 (low); 3.8 (medium)

and 7.6 (high) mg/L. After spiking of a high teicoplanin concentration to the pool of healthy plasma and HD plasma, a comparable %PB was obtained, *i.e.* 94.7 and 94.6 %, respectively and after spiking with a low concentration of teicoplanin, the %PB was in the same line, *i.e.* 94.4 %. After spiking with a medium concentration of teicoplanin, a small increase to 95.5 % was observed.

Besides a small, but significant, decrease in total concentration (-2.6 % for IAA, -2.5 % for IS and -2.9 % for *p*CS; all within the analysis variation) in HD plasma samples spiked with a low concentration of teicoplanin as compared to in HD reference samples, no change in total and free concentration was observed with increasing concentrations of teicoplanin. Consequently, the %PB of HA, IAA, IS and *p*CS remained unchanged.

5.3.5 Discussion

In this study, the %PB of vancomycin and teicoplanin as well as of a selected panel of PBUTs (*i.e.* HA, IAA, IS and *p*CS) was studied before (PBUTs only) and after spiking a pool of healthy plasma and a pool of HD plasma with either vancomycin or teicoplanin. The main findings of this *in vitro* work are: (i) for both vancomycin and teicoplanin, there was no difference in %PB between healthy and HD plasma when spiked with a high concentration; (ii) for vancomycin, there was an increase in %PB in HD plasma when spiked with a high concentration *versus* a low concentration, whereas for teicoplanin, the %PB remained unchanged, or the change was very small; (iii) for all four studied PBUTs, an increase in %PB was observed when HD plasma was spiked with a high concentration of vancomycin as compared to low concentrations and for IS and *p*CS as compared to the reference samples as well while (iv) the %PB of all four PBUTs remained unchanged when healthy and HD plasma was spiked with different concentrations of teicoplanin.

The %PB of vancomycin found in our study ranged between 50 and 60 % in both plasma pools. This is higher as compared to the findings of Sun *et al.* who reported a %PB of around 25 % after spiking to a solution of commercial human serum albumin [64]. Although the present work was performed *in vitro*, which may not completely reflect the physiologic conditions, in different *in vivo* studies, a %PB of vancomycin of 50 – 60 % has been reported as well.

Table 5-5 Total (C_T) and free (C_F) concentrations as well as the percentage protein binding (%PB) of vancomycin (vanco), hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate in a pool of plasma obtained from either healthy subjects or from patients on hemodialysis (HD) before (reference samples) and after spiking with vancomycin.

	Healthy plasma pool		HD plasma pool			
	Reference	**High Vanco**	**Reference**	**Low Vanco**	**Medium Vanco**	**High Vanco**
Vanco (mg/L)						
C_T	-	89.5[87.9;90.6]	-	21.9[21.7;22.5]	41.8[41.4;42.2]$^{+}$	88.3[87.9;88.9]$^{+\S}$
C_F	-	36.3[35.4;37.4]	-	10.7[10.5;10.8]	19.3[18.1;20.1]$^{+}$	35.6[35.2;35.8]$^{+\S}$
%PB	-	58.9[57.5;60.6]	-	50.4[49.5;51.3]	54.7[52.5;55.3]	60.0[58.8;60.4]$^{+\S}$
PBUTs (mg/dL)						
HA						
C_T	0.16[0.16;0.16]	0.19[0.18;0.19]^{o}	4.50[4.47;4.56]	4.47[4.40;4.49]	4.42[4.41;4.45]	4.44[4.44;4.50]
C_F	0.08[0.08;0.09]	0.07[0.07;0.07]^{o}	2.32[2.30;2.33]	2.40[2.36;2.44]	2.22[2.21;2.23]$^{+}$	2.23[2.19;2.24]$^{+}$
%PB	48.3[46.2;50.1]	64.6[60.7;64.8]^{o}	48.9[47.9;49.4]	45.9[45.0;46.9]	49.7[49.4;50.3]	50.2[49.5;51.3]$^{+}$
IAA						
C_T	0.05[0.05;0.05]	0.04[0.04;0.04]	0.24[0.24;0.25]	0.24[0.24;0.25]	0.24[0.24;0.24]	0.24[0.24;0.24]
C_F	0.004[0.004;0.004]	0.004[0.004;0.004]	0.08[0.07;0.08]	0.08[0.08;0.08]	0.07[0.07;0.07]$^{+}$	0.07[0.07;0.07]$^{+}$
%PB	91.6[91.4;91.7]	91.4[91.2;91.5]	69.0[68.7;69.3]	67.2[66.8;67.7]	69.5[69.3;70.3]	70.5[70.3;70.6]$^{+}$
IS						
C_T	0.09[0.09;0.09]	0.08[0.08;0.08]	1.93[1.91;1.94]	1.92[1.89;1.94]	1.92[1.91;1.92]	1.91[1.90;1.93]
C_F	< LOQ[1]	< LOQ[1]	0.11[0.11;0.12]	0.12[0.12;0.12]	0.10[0.10;0.10]	0.10[0.10;0.10]$^{o+}$
%PB	n.a.	n.a.	94.1[93.9;94.2]	93.9[93.8;93.9]	94.9[94.5;95.0]	95.0[94.9;95.0]$^{o+}$
***p*CS**						
C_T	0.20[0.20;0.21]	0.19[0.19;0.19]	3.25[3.22;3.27]	3.25[3.20;3.27]	3.21[3.19;3.23]	3.19[3.18;3.22]
C_F	< LOQ[2]	< LOQ[2]	0.19[0.18;0.20]	0.20[0.19;0.20]	0.17[0.17;0.18]	0.17[0.17;0.17]$^{o+}$
%PB	n.a.	n.a.	94.2[94.0;94.3]	93.9[93.8;94.1]	94.5[94.3;94.8]	94.7[94.7;94.8]^{o}

PBUTs: protein-bound uremic toxins; LOQ: limit of quantification; n.a.: not applicable; [1] LOQ (IS) = 0.0015 mg/dL; [2] LOQ (*p*CS) = 0.02 mg/dL.
Values (n = 6) are expressed as median [25th percentile (pct); 75th pct].
^{o} $p < 0.05$ *versus* reference sample; $^{+}$ $p < 0.05$ *versus* low vanco; § $p < 0.05$ *versus* medium vanco.

Table 5-6 Total (C_T) and free (C_F) concentrations as well as the percentage protein binding (%PB) of teicoplanin (teico), hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate in a pool of plasma obtained from either healthy subjects or from patients on hemodialysis (HD) before (reference samples) and after spiking with teicoplanin.

	Healthy plasma pool		HD plasma pool			
	Reference	**High Teico**	**Reference**	**Low Teico**	**Medium Teico**	**High Teico**
Teico (mg/L)						
C_T	-	138.1[128.8;148.1]	-	39.5[38.8;41.9]	86.2[84.2;88.1]$^{+}$	145.8[141.0;150.3]$^{+\S}$
C_F	-	7.5[7.1;7.7]	-	2.2[2.0;2.5]	3.8[3.2;4.7]$^{+}$	7.6[6.2;8.4]$^{+\S}$
%PB	-	94.7[94.0;94.9]	-	94.4[93.7;95.4]	95.5[94.6;96.2]$^{+}$	94.6[94.0;95.5]
PBUTs (mg/dL)						
HA						
C_T	0.06[0.05;0.06]	0.06[0.06;0.06]	4.25[4.22;4.26]	4.22[4.19;4.24]	4.31[4.25;4.32]	4.21[4.20;4.26]
C_F	< LOQ[1]	< LOQ[1]	2.45[2.41;2.48]	2.43[2.35;2.49]	2.40[2.38;2.41]	2.49[2.44;2.55]
%PB	n.a.	n.a.	41.8[41.4;44.4]	42.7[40.7;44.6]	44.3[43.5;45.8]	41.1[39.6;42.0]
IAA						
C_T	0.04[0.04;0.04]	0.04[0.04;0.04]	0.23[0.23;0.24]	0.23[0.23;0.23]^{o}	0.23[0.23;0.23]	0.23[0.23;0.23]
C_F	0.001[0.001;0.001]	0.001[0.001;0.001]	0.08[0.08;0.08]	0.08[0.08;0.08]	0.08[0.08;0.08]	0.08[0.08;0.08]
%PB	97.5[97.3;97.8]	97.3[96.9;97.6]	65.1[64.4;66.7]	65.0[63.8;66.3]	65.9[65.2;66.6]	64.9[63.8;65.3]
IS						
C_T	0.08[0.08;0.08]	0.08[0.08;0.08]	1.82[1.81;1.84]	1.78[1.75;1.78]^{o}	1.80[1.79;1.81]	1.78[1.77;1.80]
C_F	0.003[0.002;0.003]	0.003[0.003;0.003]	0.19[0.19;0.20]	0.18[0.18;0.19]	0.18[0.18;0.19]	0.19[0.18;0.19]
%PB	96.6[96.2;96.7]	96.5[96.4;96.7]	89.4[89.0;89.9]	89.4[89.2;89.7]	89.9[89.8;90.1]	89.6[89.5;89.8]
***p*CS**						
C_T	0.18[0.18;0.18]	0.18[0.18;0.19]	3.12[3.11;3.14]	3.03[2.99;3.04]^{o}	3.07[3.04;3.08]	3.03[3.01;3.07]
C_F	< LOQ[2]	< LOQ[2]	0.30[0.30;0.32]	0.30[0.29;0.30]	0.30[0.29;0.30]	0.30[0.29;0.30]
%PB	n.a.	n.a.	90.2[89.9;90.6]	90.1[89.9;90.5]	90.4[90.3;90.6]	90.3[90.1;90.4]

PBUTs: protein-bound uremic toxins; LOQ: limit of quantification; n.a.: not applicable [1] LOQ (HA) = 0.04 mg/dL; [2] LOQ (*p*CS) = 0.02 mg/dL.
Values (n = 6) are expressed as median [25th percentile (pct); 75th pct].
^{o} $p < 0.05$ *versus* reference sample; $^{+}$ $p < 0.05$ *versus* low teico; § $p < 0.05$ *versus* medium teico.

In vivo, the inter-patient variability in %PB is large as demonstrated by Stove *et al.* in patients from the intensive care unit (median: 25 %, range: 13 – 41 %) [76] and by Oyaert *et al.* in patients from the intensive care unit (median: 38 %, range: 12.4 – 53 %), hematology (median: 39 %, range: 9 – 51 %), orthopedics (median: 44 %, range: 22 – 54 %) and pediatrics (median: 19 %, range: 4 – 38%) ward [69]. Butterfield *et. al.* also reported a large variation in %PB of vancomycin (median: 41 %, range: 24 – 64 %) in patients from the intensive care unit, the dermatology and pneumology ward [67].

Because of the differences in patient populations, it is not evident to compare our data to those mentioned above. Even the comparison with two studies in which the %PB was studied in patients with CKD is not obvious [63, 64]. In the first study, Rodvold *et al.* studied the %PB of vancomycin in patients with various degrees of kidney failure. Ultrafiltration was performed at 25 °C and the %PB was found to be around 30 %, irrespective of kidney function. In the second study, Tan *et al.* reported a %PB of around 18.5 % in patients with end-stage kidney disease [49]. Here again, ultrafiltration was performed at 25 °C.

Nevertheless, one of the aims of this study was to compare the %PB of vancomycin in a plasma pool from healthy subjects *versus* HD patients. We found that the %PB was not affected by renal failure, which is in line with the conclusion of Rodvold *et al*. where the %PB remained unchanged at different degrees of kidney failure. Our findings and those from Rodvold *et al*. are in contrast with those from Tan *et al.*, where a decrease in %PB of vancomycin was reported from 46 % in pooled serum from healthy subjects with normal renal function to 18.5 % in patients with end-stage kidney disease. Besides vancomycin, other basic drug compounds were previously studied in patients with renal failure. For these drugs, the %PB was found to be either increased (e.g. clonidine and propranolol [6]) or unchanged (e.g. penbutolol [57] and gentamicin [58]). Thus, our findings are in line with those of other basic drug compounds.

The here reported %PB of 95 % for teicoplanin is comparable to the range reported in the literature, *i.e.* 90 % – 97 % [65, 70–72]. To the best of our knowledge, this paper is the first to compare the %PB of teicoplanin in plasma from healthy volunteers versus HD patients. In accordance to our findings when spiking with a high concentration of vancomycin, also for teicoplanin the %PB does not seem to be affected in CKD.

Measured PBUT concentrations are in line with those previously described in HD patients [22, 23, 26, 46, 77]. While free PBUT concentrations and %PB were not changed after spiking with different concentrations of teicoplanin, changes in %PB were observed after spiking with a high

concentration of vancomycin. Although the decrease in free fraction concentrations was between 7 and 18 %, the increase in %PB was < 9.4 % for HA, < 4.8 % for IAA and < 1.1 % for IS and *p*CS. In our opinion, this degree of change might be of limited biological relevance.

The main plasma binding protein for the four studied PBUTs is albumin and all four predominantly bind to the Sudlow II binding site [25, 44]. Recently, we demonstrated that the albumin binding sites, in the presence of uremic concentrations of HA, IAA, IS and *p*CS are far from being saturated with ligands [78]. Albumin is also the main binding protein for teicoplanin [79], whereas for vancomycin, both albumin and immunoglobulin A seem to be involved [64, 69]. In our healthy and HD plasma pools, the molar albumin concentrations were about 690 µmol/L and 574 µmol/L, respectively. This means that the median molar concentration of the PBUTs (HA: 250 µmol/L, IAA: 14 µmol/L, IS: 90 µmol/L and *p*CS: 170 µmol/L) as well of vancomycin (high: 60 µmol/L) and teicoplanin (high: 75 µmol/L) were all below that of albumin. Therefore, it was not surprising that the %PB of PBUTs, vancomycin and teicoplanin was not affected when all compounds were present in the same solution. In addition, vancomycin and teicoplanin could also bind to another site on albumin than PBUTs do. Information supporting this hypothesis is, to the best of our knowledge, not available.

In a next step, the %PB of both antibiotics could be studied in patients to whom either vancomycin or teicoplanin was administered and compared with the %PB in patients with normal renal function to see whether our *in vitro* data could be translated to *in vivo* conditions. In this way, also the variability in %PB of vancomycin and teicoplanin could be further explored in these two patient populations.

5.3.6 Conclusion

Percentage protein binding of both vancomycin and teicoplanin is not affected in plasma of HD patients *versus* healthy controls. In addition, no relevant biological changes in the percentage protein binding of hippuric acid, indole-3-acetic acid, indoxyl sulfate and *p*-cresyl sulfate were observed, which may imply that both antibiotics bind at a different binding site on albumin and/or the number of binding sites on albumin are not saturated in the studied clinically relevant concentration ranges.

5.3.7 Acknowledgements

This project was supported by The Research Foundation – Flanders (FWO Vlaanderen, Project Number G0A4614N).

5.4 References

1. Bertuzzi A, Mingrone G, Gandolfi A, Greco A V., Ringoir S, Vanholder R (1997) Binding of indole-3-acetic acid to human serum albumin and competition with L-tryptophan. Clin Chim Acta 265:183–192.

2. Mingrone G, De Smet R, Greco AV, Bertuzzi A, Gandolfi A, Ringoir S, Vanholder R (1997) Serum uremic toxins from patients with chronic renal failure displace the binding of L-tryptophan to human serum albumin. Clin Chim Acta 260:27–34. doi: 10.1016/S0009-8981(96)06504-7

3. Bergé-Lefranc D, Chaspoul F, Cérini C, Brunet P, Gallice P (2013) Thermodynamic study of indoxylsulfate interaction with human serum albumin and competitive binding with p-cresylsulfate. J Therm Anal Calorim 115:2021–2026. doi: 10.1007/s10973-013-3067-6

4. Klammt S, Wojak HJ, Mitzner A, Koball S, Rychly J, Reisinger EC, Mitzner S (2012) Albumin-binding capacity (ABiC) is reduced in patients with chronic kidney disease along with an accumulation of protein-bound uraemic toxins. Nephrol Dial Transplant 27:2377–2383. doi: 10.1093/ndt/gfr616

5. Perucca E (1980) Plasma Protein Binding of Phenytoin in Health and Disease: Relevance to Therapeutic Drug Monitoring. Ther Drug Monit 2:331–344.

6. Vanholder R, Van Landschoot N, De Smet R, Schoots A, Ringoir S (1988) Drug protein binding in chronic renal failure: evaluation of nine drugs. Kidney Int 33:996–1004. doi: 10.1038/ki.1988.99

7. Mabuchi H, Nakahashi H (1988) Displacement by Anionic Drugs of Endogenous Ligands Bound to Albumin in Uremic Serum. Ther Drug Monit 10:261–264.

8. Takamura N, Maruyama T, Otagiri M (1997) Effects of uremic toxins and fatty acids on serum protein binding of furosemide: Possible mechanism of the binding defect in uremia. Clin Chem 43:2274–2280.

9. Dreisbach AW, Lertora JJ (2008) The Effect of Chronic Renal Failure on Drug Metabolism and Transport. Expert Opin Drug Metab Toxicol 4:1065–1074. doi: 10.1517/17425255.4.8.1065

10. Johannessen Landmark C, Johannessen SI, Tomson T (2012) Host factors affecting antiepileptic drug delivery-Pharmacokinetic variability. Adv Drug Deliv Rev 64:896–910. doi: 10.1016/j.addr.2011.10.003

11. Zaidi N, Ahmad E, Rehan M, Rabbani G, Ajmal MR, Zaidi Y, Subbarao N, Khan RH (2013) Biophysical Insight into Furosemide Binding to Human Serum Albumin : A Study To Unveil Its Impaired Albumin Binding in Uremia. doi: 10.1021/jp3069877

12. Tao X, Thijssen S, Kotanko P, Ho C, Henrie M, Stroup E, Handelman G (2016) Improved dialytic removal of protein-bound uraemic toxins with use of albumin binding competitors : an in vitro human whole blood study. Nat Publ Gr 2–10. doi: 10.1038/srep23389

13. Vanholder R, Meert N, Schepers E, Glorieux G (2008) Uremic toxins: do we know enough to explain uremia? Blood Purif 26:77–81. doi: 10.1159/000110570

14. Vanholder R, Baurmeister U, Brunet P, Cohen G, Glorieux G, Jankowski J (2008) A Bench to Bedside View of Uremic Toxins. J Am Soc Nephrol 19:863–870. doi: 10.1681/ASN.2007121377

15. Vanholder R, Van Laecke S, Glorieux G (2008) What is new in uremic toxicity? Pediatr Nephrol 23:1211–1221.

16. Vanholder R, Massy Z, Argiles A, Spasovski G, Verbeke F, Lameire N (2005) Chronic kidney disease as cause of cardiovascular morbidity and mortality. Nephrol Dial Transplant 20:1048–1056. doi: 10.1093/ndt/gfh813

17. Sarnak MJ (2003) Kidney Disease as a Risk Factor for Development of Cardiovascular Disease: A Statement From the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation 108:2154–2169. doi: 10.1161/01.CIR.0000095676.90936.80

18. Neirynck N, Vanholder R, Schepers E, Eloot S, Pletinck A, Glorieux G (2013) An update on uremic toxins. Int Urol Nephrol 45:139–150. doi: 10.1007/s11255-012-0258-1

19. Jourde-Chiche N, Dou L, Cerini C, Dignat-George F, Brunet P (2011) Vascular Incompetence in Dialysis Patients-Protein-Bound Uremic Toxins and Endothelial Dysfunction. Semin Dial 24:327–337. doi: 10.1111/j.1525-139X.2011.00925.x

20. Sirich TL, Meyer TW, Gondouin B, Brunet P, Niwa T (2014) Protein-Bound Molecules: A Large Family With a Bad Character. Semin Nephrol 34:106–117. doi: 10.1016/j.semnephrol.2014.02.004

21. Vanholder R, Schepers E, Pletinck A, Nagler E, Glorieux G (2014) The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review. J Am Soc Nephrol 1–11. doi: 10.1681/ASN.2013101062

22. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clarck W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jörres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W (2003) Review on uremic toxins: Classification, concentration and interindividual variability. Am J Soc Nephrol 63:1934–1943. doi: 10.1046/j.1523-1755.2003.00924.x

23. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A (2012) Normal and Pathologic Concentrations of Uremic Toxins. J Am Soc Nephrol 23:1258–1270. doi: 10.1681/ASN.2011121175

24. Meijers BKI, Bammens B, Verbeke K, Evenepoel P (2008) A Review of Albumin Binding in CKD. Am J Kidney Dis 51:839–850. doi: 10.1053/j.ajkd.2007.12.035

25. Viaene L, Annaert P, De Loor H, Poesen R, Evenepoel P, Meijers B (2013) Albumin is the main plasma binding protein for indoxyl sulfate and p-cresyl sulfate. Biopharm Drug Dispos 34:165–175. doi: 10.1002/bdd.1834

26. Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S (2015) Exploring Protein Binding of Uremic Toxins in Patients with Different Stages of Chronic Kidney Disease and during Hemodialysis. Toxins (Basel) 7:3933–3946. doi: 10.3390/toxins7103933

27. Krieter DH, Korner T, Devine E, Ruth M, Jankowski J, Wanner C, Lemke HD (2014) Pilot Trial on Ionic Strength Hemodiafiltration, A Novel Dialysis Technique for Increased Protein Bound Toxin Removal. Nephrol Dial Transplant 29:211–212.

28. Devine E, Krieter DH, Rüth M, Jankovski J, Lemke HD (2014) Binding affinity and capacity for the uremic toxin indoxyl sulfate. Toxins (Basel) 6:416–430. doi: 10.3390/toxins6020416

29. Mera K, Anraku M, Kitamura K, Nakajou K, Maruyama T, Tomita K, Otagiri M (2005) Oxidation and carboxy methyl lysine-modification of albumin: possible involvement in the progression of oxidative stress in hemodialysis patients. Hypertens Res 28:973–80. doi: 10.1291/hypres.28.973

30. Oettl K, Birner-Gruenberger R, Spindelboeck W, Stueger HP, Dorn L, Stadlbauer V, Putz-Bankuti C, Krisper P, Graziadei I, Vogel W, Lackner C, Stauber RE (2013) Oxidative albumin damage in chronic liver failure: Relation to albumin binding capacity, liver dysfunction and survival. J Hepatol 59:978–983. doi: 10.1016/j.jhep.2013.06.013

31. Prakash M, Phani NM, Kavya R, Supriya M (2010) Paraoxonase: Its antiatherogenic role in chronic renal failure. Indian J Nephrol 20:9–14. doi: 10.4103/0971-4065.62088

32. Thornalley PJ, Argirova M, Ahmed N, Mann VM, Argirov O, Dawnay a. (2000) Mass spectrometric monitoring of albumin in uremia. Kidney Int 58:2228–2234. doi: 10.1046/j.1523-1755.2000.00398.x

33. Thornalley PJ, Rabbani N (2009) Highlights and hotspots of protein glycation in end-stage renal disease. Semin Dial 22:400–404. doi: 10.1111/j.1525-139X.2009.00589.x

34. Zheng C-M, Ma W-Y, Wu C-C, Lu K-C (2012) Glycated albumin in diabetic patients with chronic kidney disease. Clin Chim Acta 413:1555–1561. doi: 10.1016/j.cca.2012.04.025

35. Berg A, Drechsler C, Wenger J, Buccafusca R, Hod T, Kalim S, Ramma W, Parikh S, Steen H, Friedman D, Danziger J, Wanner C, Thadhani R, Karumanchi SA (2012) Carbamylation of Serum Albumin as a Risk Factor for Mortality in Patients with Kidney Failure. 127:358–366. doi: 10.1016/j.jsbmb.2011.07.002.Identification

36. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Hörkkö S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL, Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Ho S, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL (2007) Protein carbamylation links inflammation, smoking, uremia and atherogenesis. Nat Med 13:1176–1184. doi: 10.1038/nm1637

37. Jaisson S, Larreta-Garde V, Bellon G, Hornebeck W, Garnotel R, Gillery P (2007) Carbamylation differentially alters type I collagen sensitivity to various collagenases. Matrix Biol 26:190–196. doi: 10.1016/j.matbio.2006.10.008

38. Rueth M, Lemke H-D, Preisinger C, Krieter D, Theelen W, Gajjala P, Devine E, Zidek W, Jankowski J, Jankowski V (2015) Guanidinylations of albumin decreased binding capacity of hydrophobic metabolites. Acta Physiol 215:13–23. doi: 10.1111/apha.12518

39. Davilas A, Koupparis M, Macheras P, Valsami G (2006) In-vitro study on the competitive binding of diflunisal and uraemic toxins to serum albumin and human plasma using a potentiometric ion-probe technique. J Pharm Pharmacol 58:1467–1474. doi: 10.1211/jpp.58.11.0007

40. de Loor H, Meijers BKI, Meyer TW, Bammens B, Verbeke K, Dehaen W, Evenepoel P (2009) Sodium octanoate to reverse indoxyl sulfate and p-cresyl sulfate albumin binding in uremic and normal serum during sample preparation followed by fluorescence liquid chromatography. J Chromatogr A 1216:4684–4688. doi: 10.1016/j.chroma.2009.04.015

41. Bergé-Lefranc D, Chaspoul F, Calaf R, Charpiot P, Brunet P, Gallice P (2010) Binding of p-Cresylsulfate and p-cresol to human serum albumin studied by microcalorimetry. J Phys Chem B 114:1661–1665. doi: 10.1021/jp9059517

42. Watanabe H, Noguchi T, Miyamoto Y, Kadowaki D, Kotani S, Nakajima M, Miyamura S, Ishima Y, Otagiri M, Maruyama T (2012) Interaction between two sulfate-conjugated uremic toxins, p-cresyl sulfate and indoxyl sulfate, during binding with human serum albumin. Drug Metab Dispos 40:1423–1428. doi: 10.1124/dmd.112.045617

43. Meijers BKI, De Loor H, Bammens B, Verbeke K, Vanrenterghem Y, Evenepoel P (2009) P-Cresyl Sulfate and Indoxyl Sulfate in Hemodialysis Patients. Clin J Am Soc Nephrol 4:1932–1938. doi: 10.2215/CJN.02940509

44. Watanabe H, Miyamoto Y, Otagiri M, Maruyama T (2011) Update on the Pharmacokinetics and Redox Properties of Protein-Bound Uremic Toxins. J Pharm Sci 100:3682–3695. doi: 10.1002/jps.22592

45. Gajjala PR, Fliser D, Speer T, Jankowski V, Jankowski J (2015) Emerging role of post-translational modifications in chronic kidney disease and cardiovascular disease. Nephrol Dial Transplant 1–11. doi: 10.1093/ndt/gfv048

46. de Loor H, Poesen R, De Leger W, Dehaen W, Augustijns P, Evenepoel P, Meijers B (2016) A liquid chromatography – tandem mass spectrometry method to measure a

selected panel of uremic retention solutes derived from endogenous and colonic microbial metabolism. Anal Chim Acta 936:149–156. doi: 10.1016/j.aca.2016.06.057

47. Banker MJ, Clark TH, Williams JA (2003) Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. J Pharm Sci 92:967–974. doi: 10.1002/jps.10332

48. Reidenberg MM, Drayer DE (1984) Alteration of Drug-Protein Binding in Renal Disease. Clin Pharmacokinet 9:18–26. doi: 10.2165/00003088-198400091-00003

49. Tan CC, Lee HS, Ti TY, Lee EJC (1990) Pharmacokinetic of Intravenous Vancomycin in Patients with End-Stage Renal Failure. Ther Drug Monit 12:29–34.

50. Sakai T, Akira T, Otagiri M (1995) Characterization of Binding Site of Uremic Toxins on Human Serum Albumin. Biol Pharm Bull 18:1755–1761. doi: 10.1248/cpb.37.3229

51. Zaidi N, Ajmal MR, Rabbani G, Ahmad E, Khan RH (2013) A Comprehensive Insight into Binding of Hippuric Acid to Human Serum Albumin: A Study to Uncover Its Impaired Elimination through Hemodialysis. PLoS One. doi: 10.1371/journal.pone.0071422

52. Brørs O, Jacobsen S (1985) pH lability in serum during equilibrium dialysis. Br J Clin Pharmacol 20:85–88.

53. Deltombe O, Dhondt A, Van Biesen W, Glorieux G, Eloot S (2017) Effect of sample temperature, pH, and matrix on the percentage protein binding of protein-bound uraemic toxins. Anal Methods 9:1935–1940. doi: 10.1039/C7AY00054E

54. Nishio T, Takamura N, Nishii R, Tokunaga J, Yoshimoto M, Kawai K (2008) Influences of haemodialysis on the binding sites of human serum albumin: Possibility of an efficacious administration plan using binding inhibition. Nephrol Dial Transplant 23:2304–2310. doi: 10.1093/ndt/gfn002

55. Ochs HR, Greenblati DJ, Kaschell HJ, Klehr U, Divoll M, Abernethy DR (1981) Diazepam kinetics in patients with renal insufficiency or hyperthyroidism. 829–832.

56. Costela JL, Jiḿenez R, Calvo R, Suarez E, Carlos R (1996) Serum protein binding of propofol in patients with renal failure or hepatic cirrhosis. Acta Anaesthesiol Scand 40:741–745. doi: 10.1111/j.1399-6576.1996.tb04521.x

57. Aguirre C, Calvo R, Rodriguez-Sasiain JM (1993) Unchanged protein binding of penbutolol in renal insufficiency : a possible role of carbamylation. Int J Clin Pharmacol 31:31–34.

58. Myers DR, DeFehr J, Bennet WM, Porter GA, Olsen GD (1978) Gentamicin binding to serum and plasma proteins. Clin Pharmacol Ther 23:356–60.

59. Somma S, Gastaldo L, Corti A (1984) Teicoplanin, a New Antibiotic from Actinoplanes teichomyceticus nov. sp. Antimicrob Agents Chemother 26:917–923.

60. Wilson APR, Griineberg RN, Neub H (1994) A critical review of the dosage of teicoplanin in Europe and the USA. Int. J. Antimicrob. Agents 4:S1-S30

61. Ziglam HM, Finch RG (2001) Limitations of presently available glycopeptides in the treatment of Gram- positive infection. Clin Microbiol Infect 7:53–65. doi: 10.1046/j.1469-0691.2001.00059.x

62. Matsumoto K, Watanabe E, Kanazawa N, Fukamizu T, Shigemi A, Yokoyama Y, Ikawa K (2016) Pharmacokinetic/pharmacodynamic analysis of teicoplanin in patients with MRSA infections. Clin Pharmacol Adv Appl 8:15–18. doi: 10.2147/CPAA.S96143

63. Rodvold KA, Blum RA, Fischer JH, Zokufa HZ, Rotschafer JC, Crossley KB, Rifes LJ, Golper A, Elzinga L, Noonan H, Anderson J, Gilbert DN, Bennett WM (1988) Vancomycin Pharmacokinetics in Patients with Various Degrees of Renal Function. Antimicrob AGENTS Chemother Conf Antimicrob Agents Chemother 32:848–852.

64. Sun H, Maderazo EG, Krusell AR (1993) Serum protein-binding characteristics of vancomycin. Antimicrob Agents Chemother 37:1132–1136. doi: 10.1128/AAC.37.5.1132

65. Dykhuizen RS, Harvey G, Stephenson N, Nathwani D, Gould IM (1995) Protein binding and serum bactericidal activities of vancomycin and teicoplanin. Antimicrob Agents Chemother 39:1842–1847. doi: 10.1128/AAC.39.8.1842

66. Berthoin K, Ampe E, Tulkens PM, Carryn S (2009) Correlation between free and total vancomycin serum concentrations in patients treated for Gram-positive infections. Int J Antimicrob Agents 34:555–60. doi: 10.1016/j.ijantimicag.2009.08.005

67. Butterfield JM, Patel N, Pai MP, Rosano TG, Drusano GL, Lodise TP (2011) Refining vancomycin protein binding estimates: identification of clinical factors that influence

protein binding. Antimicrob Agents Chemother 55:4277–82. doi: 10.1128/AAC.01674-10

68. Kratzer A, Liebchen U, Schleibinger M, Kees MG, Kees F (2014) Determination of free vancomycin, ceftriaxone, cefazolin and ertapenem in plasma by ultrafiltration: Impact of experimental conditions. J Chromatogr B Anal Technol Biomed Life Sci 961:97–102. doi: 10.1016/j.jchromb.2014.05.021

69. Oyaert M, Spriet I, Allegaert K, Smits A, Vanstraelen K, Peersman N, Wauters J, Verhaegen J, Vermeersch P, Pauwels S (2015) Factors impacting unbound vancomycin concentrations in different patient populations. Antimicrob Agents Chemother 59:7073–9. doi: 10.1128/AAC.01185-15

70. Peter A, Wilson R (2000) Clinical Pharmacokinetics of Teicoplanin. Clin Pharmacokinet 39:167–183.

71. Yano R, Nakamura T, Tsukamoto H, Igarashi T, Goto N, Wakiya Y, Masada M (2007) Variability in teicoplanin protein binding and its prediction using serum albumin concentrations. Ther Drug Monit 29:399–403. doi: 10.1097/FTD.0b013e3180690755

72. Roberts JA, Stove V, De Waele JJ, Sipinkoski B, McWhinney B, Ungerer JPJ, Akova M, Bassetti M, Dimopoulos G, Kaukonen KM, Koulenti D, Martin C, Montravers P, Rello J, Rhodes A, Starr T, Wallis SC, Lipman J (2014) Variability in protein binding of teicoplanin and achievement of therapeutic drug monitoring targets in critically ill patients: Lessons from the DALI Study. Int J Antimicrob Agents 43:423–430. doi: 10.1016/j.ijantimicag.2014.01.023

73. Matzke GR, Mcgory RW, Halstenson CE, And 2, Keane1 ' WF (1984) Pharmacokinetics of Vancomycin in Patients with Various Degrees of Renal Function. Antimicrob Agents Chemother 25:433–437.

74. Livornese LL, Slavin D, Gilbert B, Robbins P, Santoro J (2004) Use of antibacterial agents in renal failure. Infect Dis Clin North Am 18:551–579. doi: 10.1016/J.IDC.2004.04.013

75. Falcoz C, Ferry N, Pozet N, Cuisinaud G, Zech PY, Sassard1 J (1987) Pharmacokinetics of Teicoplanin in Renal Failure. Antimicrob Agents Chemother 31:1255–1262.

76. Stove V, Coene L, Carlier M, Waele JJ De, Fiers T, Verstraete AG (2015) Measuring Unbound Versus Total Vancomycin Concentrations in Serum and Plasma : Methodological Issues and Relevance. Ther Drug Monit 37:180–187.

77. Itoh Y, Ezawa A, Kikuchi K, Tsuruta Y, Niwa T (2012) Protein-bound uremic toxins in hemodialysis patients measured by liquid chromatography/tandem mass spectrometry and their effects on endothelial ROS production. Anal Bioanal Chem 403:1841–1850. doi: 10.1007/s00216-012-5929-3

78. Deltombe O, de Loor H, Glorieux G, Dhondt A, Van Biesen W, Meijers B, Eloot S (2017) Exploring binding characteristics and the related competition of different protein-bound uremic toxins. Biochimie 139:20–26. doi: 10.1016/j.biochi.2017.05.010

79. Assandri A, Bernareggi A (1987) Binding of teicoplanin to human serum albumin. Eur J Clin Pharmacol 33:191–195. doi: 10.1007/BF00544566.

Chapter 6

Selective transport of protein-bound uremic toxins in erythrocytes

6.1 Introduction

Protein-bound uremic toxin (PBUT) kinetics during hemodialysis are different as compared to the kinetics of small water-soluble solutes such as urea or creatinine [1–5] since only the free fraction can travel through the pores of a hemodialyzer membrane. Previous studies were conducted aiming to enhance their plasma clearance when passing the hemodialyzer [6–8]. For some of these PBUTs, it was previously shown that their distribution is not limited to the patient's total plasma volume [5,9]. Hence, these solutes might also be distributed in the tissues. Therefore, besides the clearance within the hemodialyzer, the rate of equilibration across cellular membranes might be an important parameter to implement in dialysis kinetic models.

In this chapter, the intracellular distribution of a selected panel of PBUTs is demonstrated in an easily accessible compartment, *i.e.* the erythrocytes. Furthermore, kinetic parameters describing the transport across the erythrocyte membrane were elucidated and the impact of an anion transport blocker was studied to better understand the transport mechanism across the erythrocyte membrane.

6.2 Selective transport of protein-bound uremic toxins in erythrocytes

Will shortly be submitted in Toxins: Deltombe O, Glorieux G, Masereeuw R, Schneditz D, Eloot S Selective transport of protein-bound uremic toxins in erythrocytes

6.2.1 Abstract

To better understand the kinetics of protein-bound uremic toxins (PBUTs) during hemodialysis (HD), we investigated the distribution of hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) in erythrocytes of HD patients. Their transport across the erythrocyte membrane was explored in absence of plasma proteins *in vitro* in a series of loading and unloading experiments of erythrocytes from healthy subjects and HD patients, respectively. Furthermore, the impact of an inhibitor (*i.e.* 4,4' diisothiocyanato-2,2'-stilbenedisulfonic acid; DIDS) of uptake by Band 3 proteins expressed on the erythrocyte membrane was studied. The four PBUTs accumulated in erythrocytes from HD patients. From loading and unloading experiments, it was found that (i) the rate of transport was dependent on the studied PBUT and increased in the following sequence: HA < IS < *p*CS < IAA and that (ii) the solute partition of intra- to extracellular concentrations was uneven at equilibrium. Finally, inhibiting Band 3 proteins affected the transport of HA, IS and *p*CS, decreasing their transport parameters to comparable values. By exploring erythrocyte transmembrane transport of PBUTs, their kinetics can be better understood, and new strategies to increase their dialysis removal can be developed.

6.2.2 Introduction

The group of protein-bound uremic toxins (PBUTs) include all uremic retention solutes binding to plasma proteins with most of them having a molecular weight < 500 Da [10,11]. The percentage that is bound to proteins (% protein binding, %PB) is dependent on the solute itself rather than on binding competition or protein saturation [12] and may reach more than 99 % [13,14]. In normal conditions, the free (*i.e.* unbound) fraction of the toxins is passively cleared by glomerular filtration, whereas organic anion and cation transporters present in renal proximal tubule cells are responsible for the active secretion of the protein-bound fraction into the urine [15,16].

In patients with end-stage kidney disease treated with hemodialysis (HD), only the fraction that is not bound to proteins can pass the pores of the hemodialyzer membrane. Consequently, the dialyzer clearance of these solutes is much lower than that for comparable small non-protein-bound solutes, especially when the %PB is high [1,2,5]. During the past decade, studies have been performed aiming to improve the removal of these PBUTs during hemodialysis. Amongst others, the removal of PBUTs was shown to be enhanced when using extended hemodialysis and hemodiafiltration [17,18], by changing the local ionic strength at the blood inlet of the dialyzer increasing the free fraction [7], and by combining dialysis with adsorption [19–22]. Besides the studies on extended HD, most of these papers only focused on the removal of PBUTs from the plasma compartment ignoring the role of erythrocytes constituting almost 30 – 40 % of the blood volume passing the dialyzer [5,9].

Small water-soluble solutes are known to distribute in plasma and erythrocytes [23] and the transport through the erythrocyte membrane has been studied before for urea, uric acid and creatinine [24–28]. For PBUTs, the distribution in erythrocytes is unknown, but may help to explain their kinetics in the patient [2,14] as well as in the hemodialyzer [14].

To address this question, the present study investigated the distribution of four different anionic PBUTs [hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS)] in erythrocytes as well as their transport across the erythrocyte membrane. Furthermore, to better understand the transport mechanism across the erythrocyte membrane, the impact of an inhibitor (*i.e.* 4,4’ diisothiocyanato-2,2’-stilbenedisulfonic acid; DIDS; SLC4A1) of anion uptake by erythrocytes [29–32] was studied.

6.2.3 Materials and methods

6.2.3.1 Sample collection

Blood samples from 6 HD patients were collected pre-dialysis to determine the presence of PBUTs in erythrocytes, while blood was sampled from 8 healthy controls and another 8 HD patients (pre-dialysis) to be used in the loading and unloading experiments, respectively. All blood was sampled in K_2EDTA plasma tubes (Becton Dickinson Company, New Jersey, USA).

This study was conducted according to the Declarations of Helsinki, was approved by the Ethics Committee of Ghent University Hospital (2017/0162) and all participants gave their written informed consent.

6.2.3.2 Chemicals

HA, IAA and IS were purchased from Sigma-Aldrich (St. Louis, MO, USA) and *p*CS was obtained from TCI Chemicals (Zwijndrecht, Belgium). Water (HPLC grade) was purchased from Acros Organics (Thermo Fisher Scientific, Geel, Belgium) and the inhibitor DIDS from Sanbio (Cayman Chemical, MI, USA). A stock solution containing HA, IAA, IS and *p*CS (further assigned as PBUT mix) as well as a DIDS stock solution were both prepared in phosphate buffered saline (PBS) buffer pH = 7.4 (Invitrogen, Thermo Fischer Scientific, Ghent, Belgium) and stored at -20 °C. Theoretical final PBUT concentrations were based on the maximum uremic levels as reported by Duranton *et al.* [11]. Therefore, stock concentrations (200 times the final concentration) of 80 mmol/L (HA), 2 mmol/L (IAA) and 20 mmol/L (IS and *p*CS) were prepared. For DIDS, a theoretical final concentration of 100 µmol/L was achieved by preparing a stock concentration (13 times the final concentration) of 1300 µmol/L.

6.2.3.3 In vitro protocols

To separate erythrocytes and plasma, blood was centrifuged for 10 min at 3000 rpm, according to 2095 *g*, at room temperature (Beckman Coulter X-15R centrifuge - VWR, Leuven, Belgium).

Proof of concept – PBUTs in erythrocytes? After blood centrifugation, plasma was removed and the PBUT concentrations were determined in the erythrocyte pellet. This pellet was lysed during the first step of the sample preparation where HPLC grade water was added, followed by heating up to 95 °C.

Transport across the erythrocyte membrane. In loading (*i.e.* influx) and unloading (*i.e.* efflux) experiments, plasma from 13 mL blood samples was replaced by an equal volume of PBS to avoid the impact of plasma protein binding on the transport. Subsequently, the obtained erythrocyte suspension was gently mixed. Only in loading experiments, the erythrocyte suspension was first incubated in a water bath at 37 °C for 1 hour before the addition of 65 µL of PBUT mix, followed by gentle mixing.

Impact of the anion transport inhibitor on influx. After centrifugation of 13 mL blood samples, plasma was removed and 1 mL DIDS solution (*i.e.* DIDS in PBS) was added to the pellet as well as PBS to obtain the original blood volume. The obtained erythrocyte suspension was gently mixed and samples were incubated in a water bath at 37 °C for 1 hour. Subsequently, 65 µL of PBUT mix was added and samples were gently mixed.

After gently mixing the erythrocyte suspension in each series of experiments, aliquots (1 mL) were incubated in a water bath at 37 °C while continuously shaken to avoid erythrocyte sedimentation. At certain time points [6 (loading only), 8, 10, 13, 16, 18, 23, 28, 38, 53, 68 min], an aliquot was removed from the water bath, immediately centrifuged (Beckman Coulter Microfuge 18 – Analis, Ghent, Belgium, 5000 rpm according to 2306 *g*, 5 min), and the obtained supernatant was stored at -80 °C until batch analysis.

6.2.3.4 Analyses

Total Total PBUT concentrations were determined by an ultra-high performance liquid chromatography instrument with ultraviolet (for HA) and fluorescence (for IAA, IS and *p*CS) detection (UHPLC-UV/FLD). Both sample preparation and chromatographic methods were previously described in more detail [33].

Hematocrit (H) was determined by transferring blood into capillary tubes before centrifugation (Hettich centrifuge – Tuttlingen, Germany, 10000 rpm according to 9503 *g*, 2 min) and was manually read on a calibrated plate in all loading and unloading experiments, in the non-treated whole blood samples as well as in the corresponding erythrocyte suspensions..

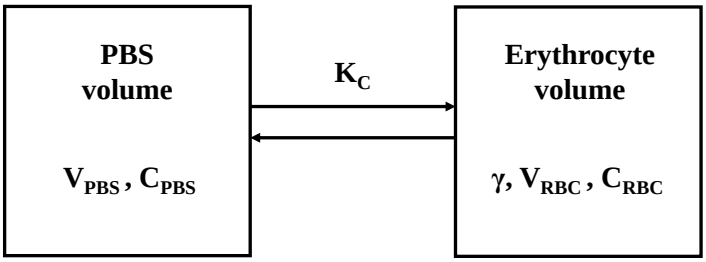
6.2.3.5 Kinetic model

The transport characteristics for a solute evenly equilibrating across intra- and extracellular compartments has been described using a two-compartment model [27]. This model, however,

needs to be adapted for solutes with non-uniform equilibration, as schematically shown in Figure 6-1. Differences in distribution can be quantified by the solute partition coefficient (γ) defined as the ratio of solute concentrations in both compartments at equilibrium [34]. In this work, those two compartments included erythrocytes (red blood cells, RBC: C_{RBC}) and PBS (C_{PBS}):

$$\gamma = \frac{C_{RBC}}{C_{PBS}} \qquad \text{(Eq. 6-1)}$$

Figure 6-1 Two-compartment model.



PBS: phosphate buffered saline; V_{PBS}: PBS water volume; C_{PBS}: solute concentration in PBS water volume; γ: solute partition coefficient; V_{RBC}: erythrocyte water volume; C_{RBC}: solute concentration in erythrocyte water volume; K_C: intercompartment clearance.

Assuming constant erythrocyte and PBS volumes, the two-compartment model for solute equilibration in a blood sample between erythrocytes and PBS is given as

$$V_{PBS}\ \frac{dC_{PBS}}{dt} = K_C\ \left(\frac{C_{RBC}}{\gamma} - C_{PBS}\right) \qquad \text{(Eq. 6-2)}$$

$$V_{RBC}\ \frac{dC_{RBC}}{dt} = -\ K_C\ \left(\frac{C_{RBC}}{\gamma} - C_{PBS}\right)$$

where V_{PBS} and V_{RBC} (both in L) refer to the volumes of PBS water and erythrocyte water (*i.e.* the cytoplasm), C_{PBS} and C_{RBC} (both in mol/L) are PBUT concentrations in PBS and erythrocyte compartments respectively, γ accounts for intracellular partition and the proportionality factor K_C (in L/min) refers to the intercompartment clearance.

The total mole n (in mol) of solute in the entire erythrocyte suspension is constant:

$$n = V_{PBS}C_{PBS} + V_{RBC}C_{RBC} \qquad \text{(Eq. 6-3)}$$

so that Eq. 6-2 can be simplified to obtain the following relationship:

$$\frac{dC_{PBS}}{dt} = \frac{K_C n}{\gamma V_{RBC} V_{PBS}} - \frac{K_C\ (\gamma V_{RBC} + V_{PBS})}{\gamma V_{RBC} V_{PBS}} C_{RBC} \qquad \text{(Eq. 6-4)}$$

The solution of this ordinary differential equation for the interval t = 0 to t = t is

$$C_{PBS(t)} = \left(C_{PBS(0)} - \frac{b}{a}\right) e^{-at} - \frac{b}{a} \quad \text{(Eq. 6-5)}$$

where the macro parameters b (in mol/min/ L) and equilibration time constant a (in 1/min) are given as

$$b = \frac{K_C n}{\gamma V_{RBC} V_{PBS}} \quad \text{(Eq. 6-6)}$$

$$a = K_C \frac{\gamma V_{RBC} + V_{PBS}}{\gamma V_{RBC} V_{PBS}} \quad \text{(Eq. 6-7)}$$

and therefore

$$\frac{b}{a} = \frac{n}{\gamma V_{RBC} + V_{PBS}} \quad \text{(Eq. 6-8)}$$

which is the equilibrated concentration ($C_{PBS(eq)}$). These equations are comparable to those as derived previously, with the difference of γ [27].

Intercompartment clearance K_C is determined by erythrocyte volume (*i.e.* erythrocyte suspension volume, V_{susp}, times hematocrit, H_{susp}) and specific rate constant k_s (1/min):

$$K_C = k_s H_{susp} V_{susp} \quad \text{(Eq. 6-9)}$$

Where k_s represents the specific rate constant, which is a more general measure for the diffusion rate across the erythrocyte membrane. For this parameter, the hematocrit [$H_{susp} = V_{RBC} / (V_{RBC} + V_{PBS})$] and water fractions for PBS ($f_{PBS} = V_{PBS,w} / V_{PBS}$) and erythrocyte ($f_{RBC} = V_{RBC,w} / V_{RBC}$) compartments are introduced because blood is usually measured as bulk volumes:

$$k_s = \frac{a}{\frac{1}{f_{PBS}}\left(\frac{H_{susp}}{1-H_{susp}}\right) + \frac{1}{\gamma f_{RBC}}} \quad \text{(Eq. 6-10)}$$

In loading experiments without inhibitor, the model parameters k_s and γ were identified by fitting the function in Eq. 6-5 and the macro parameters a (Eq. 6-7) and $C_{PBS(eq)}$ (= b/a, Eq. 6-8) to experimental data: $C_{PBS(t)}$, H_{susp}, mass of the erythrocyte suspension and concentration as well as volume of the added PBUT mix. In loading experiments in presence of inhibitor, the model parameter k_s was identified according to the same procedure, whereas the individual value for γ was assumed to be the same as in loading experiments without inhibitor and was therefore fixed for each healthy subject. In unloading experiments, mean values for γ as obtained in loading experiments without inhibitor were used to identify k_s and the following

experimental data were used to fit the function in equation 6-5 and the macro parameters a (Eq. 6-7) and $C_{PBS(eq)}$ (= b/a, Eq. 6-8): $C_{PBS(t)}$, H_{susp}, mass of the erythrocyte suspension and the measured PBUT concentration after 1 hour (*i.e.* $C_{PBS(eq)}$,m). In each series of experiments, water fractions in plasma and erythrocytes were assumed as $f_{PBS} = 0.99$ and $f_{RBC} = 0.70$ and Berkeley Madonna software (University of California, Berkeley, CA, USA) was used for parameter identification. The source codes for loading and unloading experiments are provided in 6.2.9 Supplementary material. Two Berkeley Madonna model files including experimental data, for representative loading and unloading experiments, are available as supplementary digital material.

6.2.3.6 Statistics

Statistical evaluation was performed with GraphPad Prism 4.00 for Windows (GraphPad Software, La Jolla, CA, USA) and data were checked for normality (Shapiro-Wilk test). Normally distributed data are presented as mean ± standard deviation, whereas non-normal data are presented as median [25th percentile (pct); 75th pct]. Paired samples T-tests and Mann-Whitney tests as well as repeated measures ANOVA tests and Friedman tests with Tukey (ANOVA test) or Dunns (Friedman tests) post hoc analysis were used when appropriate. $P < 0.05$ was considered significant.

6.2.4 Results

Presence of PBUTs in erythrocytes. In pre-dialysis blood from HD patients, it appeared that HA, IAA, IS and *p*CS are present in erythrocytes as determined by their concentration in the cell pellets *viz.* 88.1 [22.0;239.1], 2.4 [1.8;2.9], 29.7 [15.9;33.9] and 25.8 [18.4;30.6] µmol/L, respectively.

Experimental data for loading and unloading experiments. For each series of experiments, we determined PBUT concentrations in the PBS supernatant, calculated immediately after spiking ($C_{PBS(0)}$,c) and measured at equilibrium ($C_{PBS(eq)}$,m) (Table 6-1). In addition, the erythrocyte suspension volume (V_{susp}), hematocrit in native whole blood sample (H_{wb}) and hematocrit in erythrocyte suspension (H_{susp}) are provided in Table 6-1.

.

Table 6-1 Experimental data for loading and unloading experiments.

		$C_{PBS(0)}$,c (µmol/L)	$C_{PBS(eq)}$,m (µmol/L)	V_{susp} (mL)	H_{wb} (%)	H_{susp} (%)
Loading				12.50 ± 0.11	47 ± 5	46 ± 5
	HA	802.9 ± 72.3	504.3 ± 33.1			
	IAA	19.2 ± 1.7	9.2 ± 0.5			
	IS	227.6 ± 20.5	92.5 ± 7.0			
	*p*CS	209.6 ± 18.9	122.9 ± 13.1			
Loading + inhibitor				12.50 ± 0.11	47 ± 5	46 ± 5
	HA	804.3 ± 76.4	518.6 ± 42.4			
	IAA	19.2 ± 1.8	8.3 ± 0.5			
	IS	228.0 ± 21.7	146.5 ± 14.5			
	*p*CS	210.0 ± 20.0	131.8 ± 12.5			
Unloading				12.69 ± 0.08	36 ± 3	36 ± 3
	HA	0	39.8 [28.8;69.6]			
	IAA	0	0.8 [0.7;1.1]			
	IS	0	7.7 [5.6;9.8]			
	*p*CS	0	11.8 [7.0;18.2]			

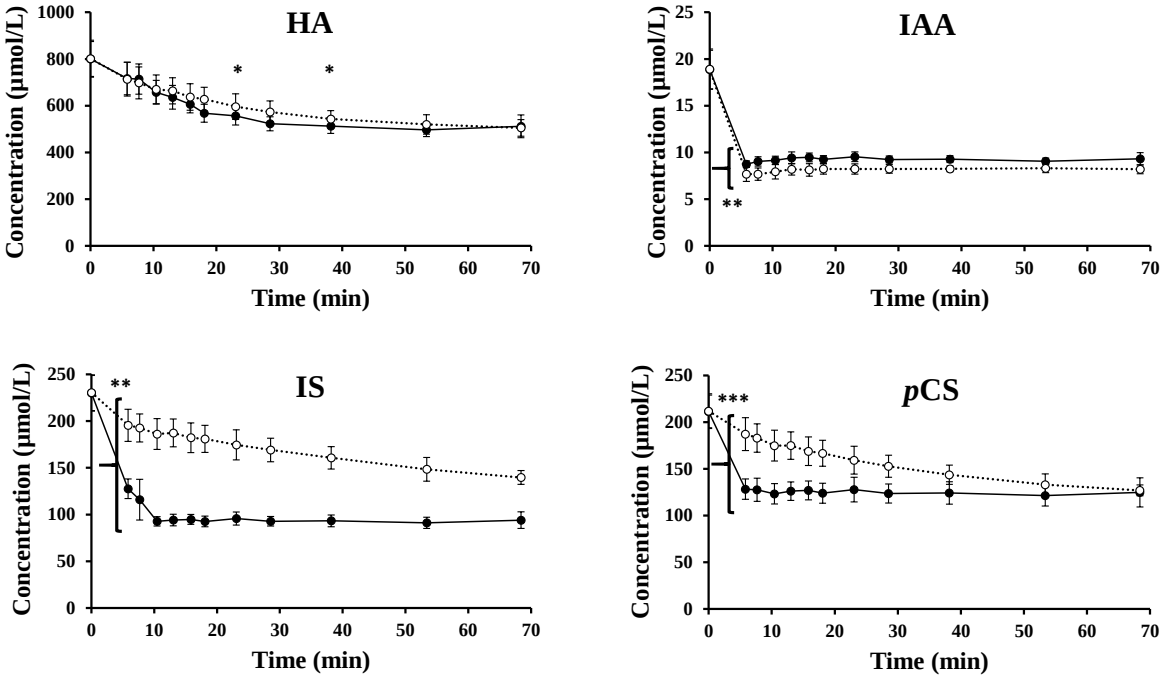
HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate; $C_{PBS(0)}$,c: calculated PBUT concentration at t = 0 min; $C_{PBS(eq)}$,m: measured PBUT concentration at equilibrium; V_{susp}: erythrocyte suspension volume; H_{wb}: hematocrit in native whole blood sample; H_{susp}: hematocrit in erythrocyte suspension. Values are mean ± standard deviation or median [25^{th} pct ;75^{th} pct]. All $n = 8$.

Transport across the erythrocyte membrane - influx. The decrease in mean HA, IAA, IS and *p*CS concentration in the PBS fraction as obtained in loading experiments of erythrocytes from healthy subjects in absence of an anion transport inhibitor is illustrated in Figure 6-2. After 6 min, this decrease was significant as compared to the theoretical start concentration for all four PBUTs and the concentrations further decreased to reach an equilibrium at 38 min for HA and 10 min for IAA and IS. For *p*CS, the concentration decreased almost immediately after spiking and equilibrium was already formed at the first experimental time point.

Impact on influx of the anion transport inhibitor. The change in mean HA, IAA, IS and *p*CS concentration in the PBS fraction as obtained during loading experiments in presence of the inhibitor DIDS is illustrated in Figure 6-2. Here again, concentrations of the four PBUTs decreased over time and reached an equilibrium at 38 min for HA. For IAA, IS and *p*CS, however, no equilibrium was formed within the time course of the experiment, indicating a slower transport in presence of the inhibitor.

The impact of DIDS is visible in the PBUT concentrations remaining lower (*i.e.* for IAA) and higher (*i.e.* for IS) in the PBS during the complete experimental time course, and for *p*CS at least up to 38 min (Figure 6-2). Overall HA transport was slightly influenced by the presence of DIDS with concentrations significant different at 25 and 40 min only.

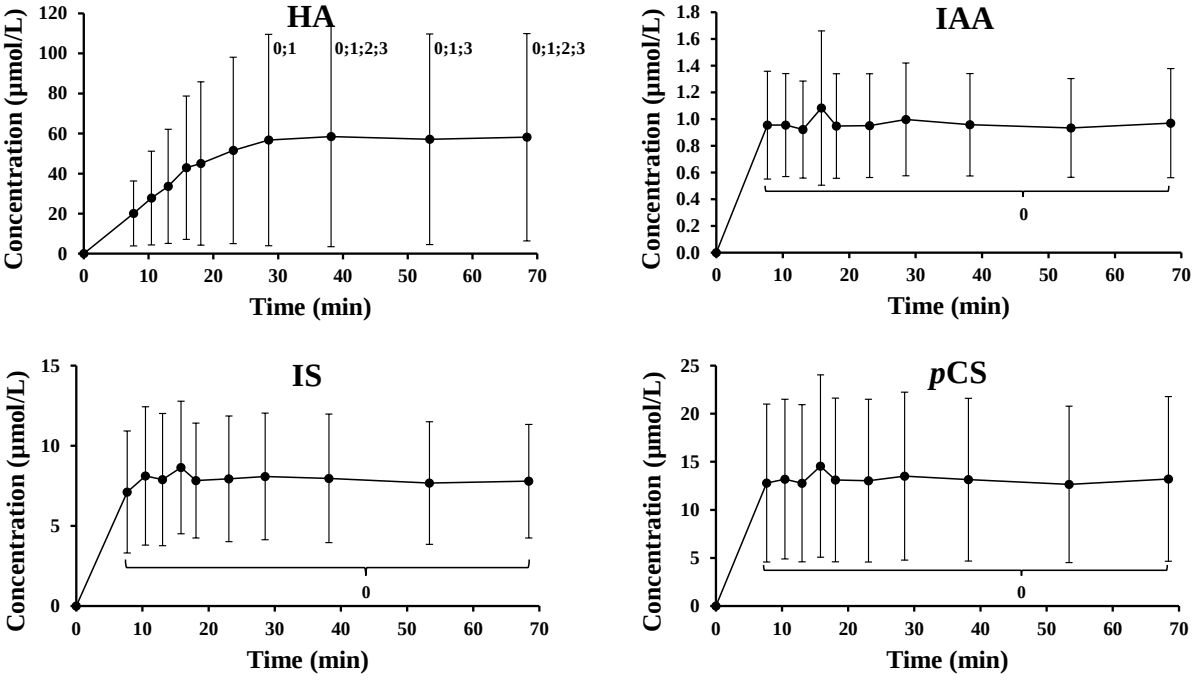
Figure 6-2 Concentration over time for hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) as measured in the PBS fraction during loading experiments in the absence (black dots and full line) and presence (white dots and dotted line) of the inhibitor DIDS, respectively.



Within each condition, the decrease in supernatant PBUT concentration was significant up to 38 min (HA), up to 10 min (IAA and IS in absence of the inhibitor) or 38 min (IAA, IS and *p*CS in presence of the inhibitor), respectively, but is not indicated on the figures for clarity.

* $p < 0.05$ *versus* absence of the inhibitor at the same time points; ** $p < 0.05$ *versus* absence of the inhibitor during the complete time course; *** $p < 0.05$ *versus* absence of the inhibitor up to 38 min. All $n = 8$.

Figure 6-3 Concentration over time for hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) as measured in the PBS fraction during unloading experiments.



[0] $p < 0.05$ *versus* t = 0 min; [1] $p < 0.05$ *versus* 8 min; [2] $p < 0.05$ *versus* 10 min; [3] $p < 0.05$ *versus* 13 min. All $n = 8$.

Transport across the erythrocyte membrane - efflux. Figure 6-3 shows the mean HA, IAA, IS and *p*CS concentrations measured in the PBS fraction as obtained in unloading experiments of erythrocytes from HD patients. Despite the large inter-patient variability, a trend in increasing HA concentration was observed. HA concentrations measured in the PBS were found significantly increased after 29 min as compared to the concentration at t = 0 and 8 min, and remained unchanged from 38 min on (Figure 6-3). For IAA, IS and *p*CS, however, this transport was very fast and corresponding concentrations were already in equilibrium at the first experimental time point.

Kinetics. Experimental data from both series of loading experiments and from unloading experiments were used to fit the following kinetic parameters for HA, IAA, IS and *p*CS: the equilibration time constant (a), specific rate constant (k_s), intercompartment clearance (K_C) and solute partition coefficient (γ) (Table 6-2).

Table 6-2 Kinetic data for loading and unloading experiments.

		a (1/min)	k_s (1/min)	K_c (mL/min)	γ
Loading					
	HA	0.06 ± 0.02	0.03 ± 0.01	0.16 ± 0.04	1.24 ± 0.20
	IAA	1.46 ± 0.02[a]	0.82 ± 0.10[a]	4.66 ± 0.23[a]	1.54 ± 0.13[a]
	IS	0.27 ± 0.07[b]	0.19 ± 0.05[b]	1.10 ± 0.27[b]	2.72 ± 0.39[a,b]
	*p*CS	0.85 ± 0.56[a,b,c]	0.40 ± 0.23[a,b,c]	2.38 ± 1.46[a,b,c]	1.30 ± 0.24[b,c]
Loading + inhibitor					
	HA	0.04 ± 0.01[d]	0.02 ± 0.01[d]	0.12 ± 0.04[d]	1.24 ± 0.20[1]
	IAA	1.47 ± 0.001[a]	0.83 ± 0.10[a]	4.68 ± 0.19[a]	1.54 ± 0.13[1,a]
	IS	0.03 ± 0.004[a,d]	0.02 ± 0.003[b,d]	0.10 ± 0.01[b,d]	2.72 ± 0.39[1,a,b]
	*p*CS	0.05 ± 0.007[b,c,d]	0.02 ± 0.004[b,d]	0.13 ± 0.02[b,d]	1.30 ± 0.24[1,b,c]
Unloading					
	HA	0.07 ± 0.01	0.04 ± 0.01[d]	0.18 ± 0.02	1.24[2]
	IAA	2.24 [0.39;2.35][a]	1.47 [0.27;1.54][a]	6.83 [1.19;7.21][a]	1.54[2]
	IS	0.31 [0.27;0.45]	0.28 [0.25;0.38][d]	1.27 [1.10;1.91]	2.72[2]
	*p*CS	0.54 [0.40;2.32][a]	0.32 [0.25;1.35][a]	1.51 [1.02;6.34][a]	1.30[2]

HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate;
a: equilibration time constant; k_s: specific rate constant; K_C: intercompartment clearance;
γ: solute partition coefficient. Values are mean ± standard deviation or median [25th pct;75th pct].
[a, b or c] $p < 0.05$ versus HA, IAA or IS, respectively as obtained within the same series of experiments.
[d] $p < 0.05$ versus loading experiments without the inhibitor, respectively.
[1] or [2] Individual respectively mean values for γ as obtained in loading experiments without the inhibitor were used to fit parameters. All $n = 8$.

In loading experiments without inhibitor, values for the solute partition coefficient ranged between 1.24 and 2.72, suggesting an asymmetrical distribution of solute in erythrocytes and PBS for each of the PBUTs. Parameters a, k_s and K_C were found to be the lowest for HA, followed by those for IS (trend only), *p*CS and IAA.

In loading experiments in the presence of an inhibitor, the transport parameters a, k_s, and K_C reached comparable low values for HA, IS and *p*CS, whereas those for IAA were not affected (Table 6-2).

In unloading experiments, a, k_s and K_C were significantly higher for IAA and *p*CS as compared to those for HA, whereas those for IS, showed a trend to be higher than those for HA but lower than those for IAA and *p*CS.

When comparing kinetic parameters a, k_s and K_C as obtained in loading experiments with those obtained in unloading experiments, only k_s was significantly different for HA and IS.

6.2.5 Discussion

In this work, the intracellular concentration of PBUTs in erythrocytes of HD patients was measured and the transport of these solutes across the erythrocyte membrane was studied *in vitro* in blood from healthy controls as well as in blood from HD patients. Furthermore, a first attempt was undertaken to determine the transport mechanism of these PBUTs across the erythrocyte membrane by using an erythrocyte Band 3 anion transport inhibitor, DIDS. The main findings are: (i) HA, IAA, IS and *p*CS are distributed in erythrocytes; (ii) the rate of transport (*i.e.* both influx and efflux) is dependent on the studied PBUT and increases in the following sequence: HA < IS < *p*CS < IAA and (iii) the presence of DIDS, which inhibits anion transport via Band 3 protein across the erythrocyte membrane, slows down the transport of HA, IS and *p*CS

The studied PBUTs distribute within the erythrocytes, which was confirmed by spiking an erythrocyte suspension with a high uremic concentration of PBUTs and measuring their uptake over time. After PBUT addition to the extracellular (PBS) compartment, concentrations in PBS decreased either slowly (*i.e.* for HA), at intermediate speed (*i.e.* for IS), or very fast (*i.e.* for *p*CS and IAA) so that an apparent equilibrium between the PBS and erythrocyte compartments was established after, respectively, 38 min, 10 min or almost immediately. Obtained transport parameters [*i.e.* equilibration time constant (a), specific rate constant (k_s) and intercompartment clearance (K_C)] demonstrated that HA is more slowly transported into erythrocytes as compared to IS (trend), *p*CS and IAA. When unloading erythrocytes from HD patients suspended in PBS, it was found that the rate of solute efflux was comparable to the rate of solute influx, based on comparable kinetic parameters between unloading and loading experiments, despite the larger inter-patient variability on both the PBUT concentrations and the kinetic parameters (not for

HA). Consequently, it seemed that the influx and efflux transport of PBUTs across the erythrocyte membrane was symmetrical, suggesting a facilitated diffusion mechanism driven by a concentration gradient.

In loading experiments in absence of an inhibitor, the solute partition coefficient (γ) for each of the studied PBUTs exceeded 1, implying an apparent equilibrium with higher concentrations in the erythrocytes as compared to in PBS. This might be due to interaction with membrane structures and/or cytoplasmic components. For the latter, we may however exclude hemoglobin since interaction with hemoglobin was found negligible in an *in vitro* experiment (n = 3) studying possible binding of these PBUTs to hemoglobin (data not shown).

It appeared that the influx and efflux of IAA across the erythrocyte cell membrane is much faster than for the other three PBUTs, which is reflected by its high values for a, k_s and K_C. Concentrations of indole, the *in vivo* precursor of IAA, were determined in some of the loading experiment samples to check whether the fast decrease in IAA concentrations was due to a fast back transformation of IAA into indole. However, indole concentrations were negligible in the PBS fraction (data not shown). Alternatively, (part of) IAA could bind to the erythrocyte membrane surface or to proteins present in the cell membrane or in the cytoplasm immediately after addition of the PBUT mix, resulting in an apparent fast removal from the PBS compartment [35]. However, to the best of our knowledge, no data of IAA binding to erythrocyte membranes is available in literature to support this hypothesis.

We also studied the impact of an inhibitor (*i.e.* DIDS) for anion transport. This compound is known to reversibly bind to Band 3 proteins, anion exchangers located on erythrocyte membranes, mediating transmembrane transport [29–32]. Because these Band 3 proteins can only influence solute transport, the distribution of PBUTs will not be changed. Hence, in the kinetic model of the loading experiments with DIDS, solute partition coefficients can be taken equal to those as found in the loading experiments without DIDS. By doing this, we observed that the transport parameters a, k_s, and K_C decreased to comparable low values for HA, IS and *p*CS. For this reason, the transport of HA, IS and *p*CS is at least in part facilitated by Band 3 proteins (*i.e.* carrier-mediated facilitated diffusion).

Notwithstanding the similar equilibration time constants for HA, *i.e.* 0.06 ± 0.02 1/min, as found in the present work and those for creatinine, *i.e.* 0.05 ± 0.01 1/min, as previously reported by Schneditz *et al.* [27], the transport mechanism of both compounds (and partly for IS and *p*CS as well) are probably not comparable, especially because of the different net charge of these

compounds at pH 7.4 (*i.e.* positive for creatinine and negative for HA, IS and *p*CS). Apart from the anion exchanger Band 3, other anion transporters may be involved in transport of the studied PBUTs as well. Amongst others, it has been shown that the multidrug resistance protein 1 (MRP 1) was present in erythrocyte membranes [36,37]. This transporter is known to efflux several sulfate conjugates of endogenous as well as of xenobiotic compounds from diverse tissues [38,39] and might thus potentially be involved in the transmembrane transport of IS and *p*CS as well. More recently, a database (http://rbcc.hegelab.org/) was created containing information on different other transport proteins present in the erythrocyte membrane [40].

The distribution of solutes within erythrocytes and the slow transport from erythrocytes to plasma has important consequences for their removal during hemodialysis. For example, for solutes slowly equilibrating across the erythrocyte membrane, the true concentration in plasma leaving the hemodialyzer is much lower than what is measured when solutes are primarily removed from plasma, and intracellular solutes remain sequestered in erythrocytes. The concentration measured in a plasma sample, however, depends on the time the blood sample is allowed to equilibrate (*i.e.* the time erythrocytes are allowed to "unload" their solutes) and on the rate of solute equilibration between plasma and erythrocytes before the blood components are separated by centrifugation. If blood is collected at the dialyzer outlet line, there is a disequilibrium between the intra- and extracellular concentrations. For solutes equilibrating fast across the erythrocyte membrane, the solute concentration as measured in the plasma could be overestimated at the time of measurement. This results in a false underestimation of the extracorporeal solute clearance, as previously demonstrated for creatinine [41]. For the present PBUTs, we also found that the time between blood sampling and centrifugation affects the serum/plasma concentration in samples collected at the dialyzer outlet line (data provided in 6.2.8 Supplementary data).

Besides the %PB, also the magnitude and rate of accumulation of PBUTs in erythrocytes may have an impact on the amount of solute removed by the dialyzer. For solutes rapidly equilibrating across the erythrocyte membrane, solute is not only cleared from the plasma compartment but also from the erythrocyte compartment and plasma and erythrocyte concentrations will be close to equilibrium at the dialyzer inlet as well as at the outlet. On the contrary, for solutes slowly equilibrating across the erythrocyte membrane, such as creatinine, the transport across the membrane should be taken into account, as described elsewhere [42]. The exact fraction of extracorporeal blood flow cleared in the dialyzer can be estimated from

dialyzer transit time, hematocrit, and specific rate constant [27] but should be adjusted by a solute partition coefficient for the studied PBUTs.

6.2.6 Conclusion

This is the first study to identify and quantify intracellular distribution of PBUTs in erythrocytes. The rate of transport (*i.e.* influx and efflux) across the erythrocyte membrane increased according to HA < IS < *p*CS < IAA. In addition, at least part of the HA, IS and *p*CS uptake by erythrocytes is attributed to the anion transporter Band 3.

6.2.7 Acknowledgements

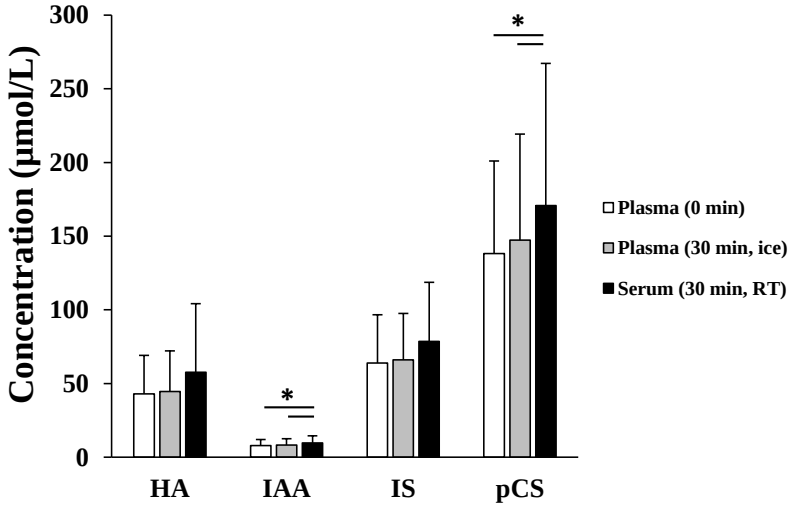
This project was supported by The Research Foundation – Flanders (FWO Vlaanderen, Project Number G0A4614N). The authors thank Sophie Lobbestael, Tom Mertens and Maria Van Landschoot for their laboratory assistance.

6.2.8 Supplementary data

6.2.8.1 Effect of time between sampling and blood centrifugation on the serum/plasma concentration of different protein-bound uremic toxins

Extra blood samples from 10 stable HD patients were collected at the dialyzer outlet line after 120 min of dialysis (performed according to the Declarations of Helsinki, approved by the Ethics Committee of Ghent University Hospital (2017/0162) and all participants gave their written informed consent). Blood was collected in two different K_2EDTA plasma tubes and in serum separating tubes (all Becton Dickinson Company, New Jersey, USA). Plasma tubes were centrifuged either immediately after sampling or after a period of 30 min on ice whereas serum tubes were left at room temperature for 30 min to allow for clotting. Total serum and plasma PBUT concentrations were determined by UHPLC-UV/FLD [36]. Statistical analysis was performed with GraphPad Prism 4.00 for Windows (GraphPad Software, La Jolla, CA, USA) and data were checked for normality (Shapiro-Wilk test). Repeated measures ANOVA and Friedman tests with, respectively Tukey and Dunns post hoc analysis, were used when appropriate. $P < 0.05$ was considered significant.

Figure 6-4 Effect of time between sampling and blood centrifugation on the serum and plasma concentrations of hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS) as determined in blood samples collected at the dialyzer outlet.



Blood tubes were centrifuged either immediately [plasma (0 min)], after 30 min on ice [plasma (30 min, ice)] or after 30 min at room temperature [(serum 30 min, RT)]. * $p < 0.05$.

Serum concentrations were significantly increased for IAA and *p*CS (Figure 6-4), while only a visual trend was observed for HA and IS in blood samples allowed to clot within 30 min at

room temperature as compared to plasma samples immediately separated after collection. Placing blood samples on ice for 30 min attenuated the equilibration process resulting in only a small increase in extracellular concentration (visual trend only).

These results indicate that when blood samples are collected the moment there is a disequilibrium between intra- and extracellular concentrations (e.g. in samples taken at the dialyzer outlet line or in samples taken at a later stage during dialysis), plasma concentrations will depend on the time between sampling and centrifugation as well as on the solute equilibration rate. Because immediate centrifugation after collection is not always achievable in clinical practice, this process of solute equilibration can be attenuated by placing blood samples immediately on ice prior to centrifugation.

6.2.9 Supplementary material

6.2.9.1 Berkeley Madonna source code for loading experiments for hippuric acid (HA) without inhibitor

```
{----------------------------------------}
{Identification of model parameters ks and gamma from equilibration in solute loading tests
using HA data from "HA_HealthyControl1(MV).txt" file using the exact analytical solution
and Berkeley-Madonna vs. 8.3. software.

Open a new file from the "File" dropdown menu and delete any default information from the
opening window.
Copy and paste the source code (from the first "{" to the last "}" of this text as plain TEXT into
that window.
Load the experimental sample data from the "Model" drop-down menu using the "Datasets"
command.
Import the "HA_HealthyControl1(MV).txt" data as 1D vector.
Run (click the "RUN" icon) this model and plot the "ct" and "#HA_HealthyControl1MVLtxt"
data vs time.
Double-click the figure and select the "#HA_HealthyControl1MVLtxt" variable for dislplay.
Then use "Curve fit" in the "Parameter" drop-down menu, select the parameters "a" and
"gamma" , and press "o.k."
The model "ct" is fit to experimental "#HA_HealthyControl1MVLtxt" data.
The parameters identified form the optimal fit can be read in the "parameter" window or by
clicking the "P" icon in the plot.
The numerical values for "ks" and Kc" and selected variables can be displayed by switching
from "plot-view" to "table view"}

STARTTIME = 0
STOPTIME = 70
DT = 0.02
```

```
Hsusp=0.425; hematocrit of erythrocyte suspension
Msusp=13.05; mass of erythrocyte suspension in g
cs=82603; concentration of HA in PBUT mix in µmol/L
Vs=0.000065; volume of spiking solution in L
fPBS=0.99; water fraction in PBS
fRBC=0.70; water fraction in erythrocytes
rhosusp=1050; erythrocyte suspension density in g/L

a=0.06; exponent, slope of the experimental decrease
gamma=1; solute partition coefficient

DISPLAY ct, a, ks, Kc, gamma

ct=(c0-ceq)*exp(-a*TIME)+ceq ; PBS concentration at time t in µmol/L
c0=ntot/(Vsusp*(1-Hsusp)*fPBS+Vs); initial PBS concentration in µmol/L
ceq=ntot/(Vsusp*(1-Hsusp)*fPBS+Vsusp*Hsusp*fRBC*gamma+Vs)
{PBS concentration at equilibrium in µmol/L}
ks=a/(Hsusp/(1-Hsusp)/fPBS+1/(gamma*fRBC)); specific rate constant in 1/min
Kc=ks*Hsusp*Vsusp*1000; intercompartment clearance in mL/min

Vsusp=Msusp/rhosusp; volume of erythrocyte sample in L
ntot=cs*Vs; total mole of solute in erythrocyte suspension in µmol
{-----------------------------------------}
```

6.2.9.2 Berkeley Madonna source code for unloading experiments for hippuric acid (HA)

```
{-----------------------------------------}
{Identification of model parameter "ks" from equilibration in solute unloading tests with
experimental HA data from "HA_ HDPatient1(AM).txt" data file using the exact analytical
solution and Berkeley-Madonna vs. 8.3. software.

Open a new file from the "File" drop-down menu and delete any default information from the
opening window.
Copy and paste the source code (from the first "{" to the last "}" of this text as plain TEXT into
that window.
Load the experimental sample data from the "Model" drop-down menu using the "Datasets"
command.
Import the "HA_ HDPatient1(AM).txt" data as 1D vector.
Run (click the "RUN" icon) this model and plot the "ct" and "#HA_ HDPatient1AMtxt" data
vs. time.
Double-click the figure and select the "#HA_ HDPatient1AMtxt" variable for dislplay.
Then use "Curve fit" in the "Parameter" drop-down menu, select parameters "a" and "ceq" and
press "o.k.".
The model "ct" is fit to experimental "#HA_ HDPatient1AMtxt" data.
The parameters identified form the optimal fit can be read in the "Parameter" window or by
clicking the "P" icon in the plot.
The numerical values for "ks" and Kc" and selected variables can be displayed by switching
from "plot-view" to "table view"}
```

```
STARTTIME = 0
STOPTIME = 70
DT = 0.02

Hsusp=0.32; hematocrit of erythrocyte suspension
Msusp=13.27; mass of erythrocyte suspension in g
ceq=70.24; measured PBS concentration at equilibrium after 1 hour in µmol/L
c0=0; solute concentration in PBS at t=0 in µmol/L
fPBS=0.99; water fraction in PBS
fRBC=0.70; water fraction in erythrocytes
rhosusp=1050; erythrocyte suspension density in g/L

a=0.06; exponent, slope of the experimental decrease
gamma=1.24; solute partition coefficient from loading experiments

DISPLAY ct, ceq, a, ks, Kc, gamma

ct=(c0-ceq)*exp(-a*TIME)+ceq; PBS concentration at time t in µmol/L
ks=a/(Hsusp/(1-Hsusp)/fPBS+1/(gamma*fRBC)); specific rate constant in 1/min
Kc=ks*Hsusp*Vsusp*1000; intercompartment clearance in mL/min
Vsusp=Msusp/rhosusp; volume of erythrocyte sample in L
{----------------------------------------}
```

6.3 References

1. Vanholder R, De Smet R, Lesaffer G (2002) Dissociation between dialysis adequacy and Kt/V. Semin Dial 15:3–7. doi: 10.1046/j.1525-139x.2002.00005.x

2. Eloot S, Van Biesen W, Dhondt A, Van de Wynkele H, Glorieux G, Verdonck P, Vanholder R (2008) Impact of hemodialysis duration on the removal of uremic retention solutes. Kidney Int 73:765–770. doi: 10.1038/sj.ki.5002750

3. Eloot S, Van Biesen W, Vanholder R (2012) A sad but forgotten truth: The story of slow-moving solutes in fast hemodialysis. Semin Dial 25:505–509. doi: 10.1111/j.1525-139X.2012.01107.x

4. Eloot S, Schneditz D, Vanholder R (2012) What can the dialysis physician learn from kinetic modelling beyond Kt/Vurea? Nephrol Dial Transplant 27:4021–4029. doi: 10.1093/ndt/gfs367

5. Eloot S, Schneditz D, Cornelis T, Van Biesen W, Glorieux G, Dhondt A, Kooman J, Vanholder R (2016) Protein-bound uremic toxin profiling as a tool to optimize hemodialysis. PLoS One 11:1–18. doi: 10.1371/journal.pone.0147159

6. Meert N, Eloot S, Waterloos MA, Van Landschoot M, Dhondt A, Glorieux G, Ledebo I, Vanholder R (2009) Effective removal of protein-bound uraemic solutes by different convective strategies: A prospective trial. Nephrol Dial Transplant 24:562–570. doi: 10.1093/ndt/gfn522

7. Böhringer F, Jankowski V, Gajjala PR, Zidek W, Jankowski J (2015) Release of Uremic Retention Solutes from Protein Binding by Hypertonic Predilution Hemodiafiltration. ASAIO J 61:55–60. doi: 10.1097/MAT.0000000000000166

8. Pavlenko D, van Geffen E, van Steenbergen MJ, Glorieux G, Vanholder R, Gerritsen KGF, Stamatialis D (2016) New low-flux mixed matrix membranes that offer superior removal of protein-bound toxins from human plasma. Sci Rep 6:34429. doi: 10.1038/srep34429

9. Maheshwari V, Thijssen S, Tao X, Fuertinger D, Kappel F, Kotanko P (2017) A novel mathematical model of protein-bound uremic toxin kinetics during hemodialysis. Sci Rep 7:10371. doi: 10.1038/s41598-017-10981-z

10. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clarck W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jörres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W (2003) Review on uremic toxins: Classification, concentration and interindividual variability. Am J Soc Nephrol 63:1934–1943. doi: 10.1046/j.1523-1755.2003.00924.x

11. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A (2012) Normal and Pathologic Concentrations of Uremic Toxins. J Am Soc Nephrol 23:1258–1270. doi: 10.1681/ASN.2011121175

12. Deltombe O, de Loor H, Glorieux G, Dhondt A, Van Biesen W, Meijers B, Eloot S (2017) Exploring binding characteristics and the related competition of different protein-bound uremic toxins. Biochimie 139:20–26. doi: 10.1016/j.biochi.2017.05.010

13. Itoh Y, Ezawa A, Kikuchi K, Tsuruta Y, Niwa T (2012) Protein-bound uremic toxins in hemodialysis patients measured by liquid chromatography/tandem mass spectrometry and their effects on endothelial ROS production. Anal Bioanal Chem 403:1841–1850. doi: 10.1007/s00216-012-5929-3

14. Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S (2015) Exploring Protein Binding of Uremic Toxins in Patients with Different Stages of Chronic Kidney Disease and during Hemodialysis. Toxins (Basel) 7:3933–3946. doi: 10.3390/toxins7103933

15. Masereeuw R, Mutsaers H, Toyohara T, Abe T, Jhawar S, Sweet DH, Lowenstein J (2014) The Kidney and Uremic Toxin Removal: Glomerulus or Tubule? Semin Nephrol 34:191–208. doi: 10.1016/j.semnephrol.2014.02.010

16. Jansen J, Jankowski J, Gajjala PR, Wetzels JFM, Masereeuw R (2017) Disposition and clinical implications of protein-bound uremic toxins. 1631–1647. doi: 10.1042/CS20160191

17. Meert N, Eloot S, Schepers E, Lemke H-D, Dhondt A, Glorieux G, Landschoot M Van, Waterloos M-A, Vanholder R (2011) Comparison of removal capacity of two consecutive generations of high-flux dialysers during different treatment modalities. Nephrol Dial Transpl 26:2624–2630. doi: 10.1093/ndt/gfq803

18. Cornelis T, Eloot S, Vanholder R, Glorieux G, Van Der Sande FM, Scheijen JL, Leunissen KM, Kooman JP, Schalkwijk CG (2015) Protein-bound uraemic toxins, dicarbonyl stress and advanced glycation end products in conventional and extended haemodialysis and haemodiafiltration. Nephrol Dial Transplant 30:1395–1402. doi: 10.1093/ndt/gfv038

19. Meijers BK, Weber V, Bammens B, Dehaen W, Verbeke K, Falkenhagen D, Evenepoel P (2008) Removal of the uremic retention solute p-cresol using fractionated plasma separation and adsorption. Artif Organs 32:214–219. doi: 10.1111/j.1525-1594.2007.00525.x

20. Brettschneider F, Tölle M, Von der Giet M, Passlick-Deetjen J, Steppan S, Peter M, Jankowski V, Krause A, Kühne S, Zidek W, Jankowski J (2013) Removal of Protein-Bound, Hydrophobic Uremic Toxins by a Combined Fractionated Plasma Separation and Adsorption Technique. Artif Organs 37:409–416. doi: 10.1111/j.1525-1594.2012.01570.x

21. Meyer TW, Peattie JWT, Miller JD, Dinh DC, Recht NS, Walther JL, Hostetter TH (2007) Increasing the Clearance of Protein-Bound Solutes by Addition of a Sorbent to the Dialysate. J Am Soc Nephrol 18:868–874. doi: 10.1681/ASN.2006080863

22. Sandeman SR, Howell CA, Phillips GJ, Zheng Y, Standen G, Pletzenauer R, Davenport A, Basnayake K, Boyd O, Holt S, Mikhalovsky S V. (2014) An adsorbent monolith device to augment the removal of uraemic toxins during haemodialysis. J Mater Sci Mater Med 25:1589–1597. doi: 10.1007/s10856-014-5173-9

23. Eloot S, Torremans A, De Smet R, Marescau B, Deyn PP De, Verdonck P, Vanholder R (2007) Complex Compartmental Behavior of Small Water-Soluble Uremic Retention Solutes : Evaluation by Direct Measurements in Plasma and Erythrocytes. Am J Kidney Dis 50:279–288. doi: 10.1053/j.ajkd.2007.05.009

24. Gary-Bobo C, Lindenberg AB (1960) Velocity of the penetration of creatinine into human erythrocytes as a function of temperature. J Physiol (Paris) 52:106–107.

25. Langsdorf LJ, Zydney AL (1993) Effect of Uremia on the Membrane Transport Characteristics of Red Blood Cells. Blood 81:820–827.

26. Sands J, Timmer R, Gunn R (1997) Urea transporters in kidney and erythrocytes. Am J Physiol 273:F321–F339.

27. Schneditz D, Yang Y, Christopoulos G, Kellner J (2009) Rate of creatinine equilibration in whole blood. Hemodial Int 13:215–221. doi: 10.1111/j.1542-4758.2009.00351.x

28. Brahm J (2013) The permeability of red blood cells to chloride, urea and water. J Exp Biol 216:2238–2246. doi: 10.1242/jeb.077941

29. Soszynski M, Bartosz G (1997) Penetration of erythrocyte membrane by peroxynitrite: participation of the anion exchange protein. Biochem Mol Biol Int 43:319–325.

30. Salhany JM (2001) Stilbenedisulfonate binding kinetics to band 3 (AE 1): Relationship between transport and stilbenedisulfonate binding sites and role of subunit interactions in transport. Blood Cells, Mol Dis 27:127–134. doi: 10.1006/bcmd.2000.0369

31. Salhany JM, Schopfer LM (2001) Kinetic mechanism of DIDS binding to band 3 (AE1) in human erythrocyte membranes. Blood Cells, Mol Dis 27:844–849. doi: 10.1006/bcmd.2001.0458

32. Reithmeier RAF, Casey JR, Kalli AC, Sansom MSP, Alguel Y, Iwata S (2016) Band 3, the human red cell chloride/bicarbonate anion exchanger (AE1, SLC4A1), in a structural context. Biochim Biophys Acta 1858:1507–1532. doi: 10.1016/j.bbamem.2016.03.030

33. Deltombe O, Dhondt A, Van Biesen W, Glorieux G, Eloot S (2017) Effect of sample temperature, pH, and matrix on the percentage protein binding of protein-bound uraemic toxins. Anal Methods 9:1935–1940. doi: 10.1039/C7AY00054E

34. Kety SS (1951) The theory and applications of the exchange of inert gas at the lungs and tissues. Pharmacol Rev 3:1–41.

35. Kakhniashvili DG, Bulla LA, Goodman SR (2004) The Human Erythrocyte Proteome ANALYSIS BY ION TRAP MASS SPECTROMETRY. Mol Cell Proteomics 3:501–509. doi: 10.1074/mcp.M300132-MCP200

36. Rychlik B, Balcerczyk A, Klimczak A, Bartosz G (2003) Membrane Biology The Role of Multidrug Resistance Protein 1 (MRP1) in Transport of Fluorescent Anions across the Human Erythrocyte Membrane. J Membr Biol 193:79–90.

37. Wesoaowska O, Mosiadz D, Motohashi N, Kawase M, Michalak K (2005) Phenothiazine maleates stimulate MRP1 transport activity in human erythrocytes. Biochim Biophys Acta 1720:52–58. doi: 10.1016/j.bbamem.2005.11.011

38. Homolya L, Váradi A, Sarkadi B (2003) Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate. BioFactors 17:103–114. doi: 10.1002/biof.5520170111

39. Deeley RG, Cole SPC (2005) Substrate recognition and transport by multidrug resistance protein 1 (ABCC1). FEBS Lett 580:1103–1111. doi: 10.1016/j.febslet.2005.12.036

40. Hegedu S TS, Chaubey PM, Rgy G, Rady V, Szabó E, Sarankó H, Hofstetter L, Roschitzki B, Stieger B, Zs Sarkadi B (2015) Inconsistencies in the red blood cell membrane proteome analysis: generation of a database for research and diagnostic applications. Database. doi: 10.1093/database/bav056

41. Descombes E, Perriard F, Fellay G (1993) Diffusion kinetics of urea, creatinine and uric acid in blood during hemodialysis. Clinical implications. Clin Nephrol 40:286–95.

42. Schneditz D, Platzer D, Daugirdas JT (2009) A diffusion-adjusted regional blood flow model to predict solute kinetics during haemodialysis. Nephrol Dial Transplant 24:2218–2224. doi: 10.1093/ndt/gfp023

Chapter 7

General summary, critical interpretation, relevance of the work and future perspectives

7.1 General summary and critical interpretation

Chronic kidney disease (CKD) is defined as either kidney damage or a glomerular filtration rate (GFR) < 60 mL/min/1.73 m² over a period of at least three months [1] (**Chapter 1**). At end-stage kidney disease (*i.e.* GFR < 15 mL/min/1.73 m²), a renal replacement therapy is often started and, besides kidney transplantation and peritoneal dialysis, hemodialysis can be initiated. In this extracorporeal technique, blood flows through the lumen of hollow fibers in an artificial kidney (*i.e.* the hemodialyzer). The semi-permeable membrane of these fibers allows the transport of solutes from the blood into a water-electrolyte fluid (*i.e.* dialysis fluid), which flows around the fibers in an opposite direction.

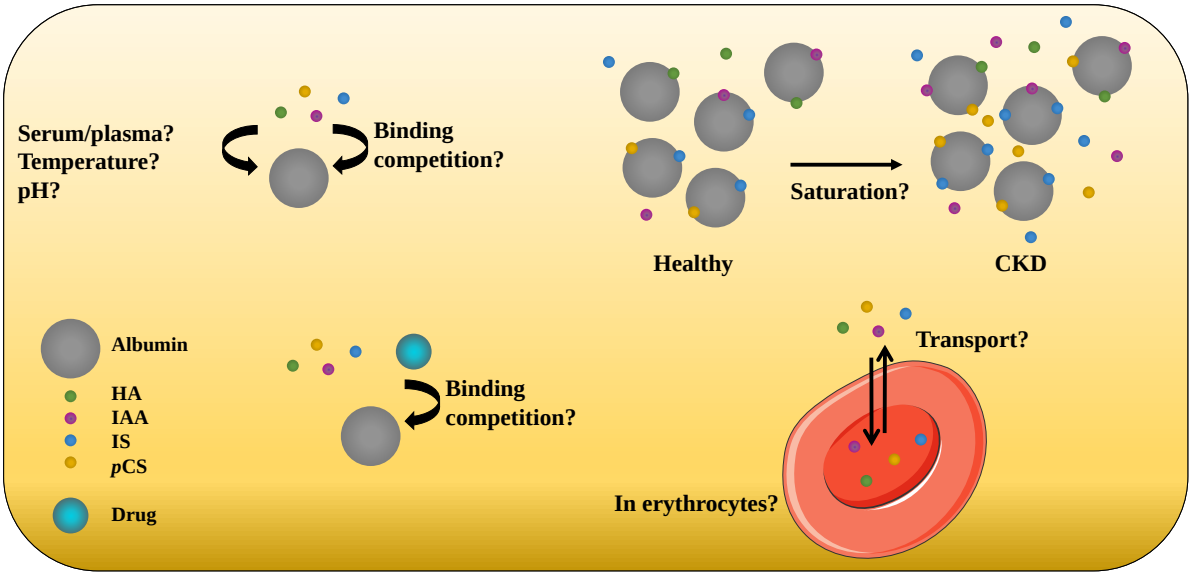
A myriad of waste products are accumulated in patients with CKD, as a result of their decreased renal function. These uremic retention solutes may exert toxicity [2–10] and many of them have an impact on the cardiovascular system and contribute thus to the increased propensity for cardiovascular events and mortality in CKD [11,12]. Based on their physico-chemical properties, the so-called uremic toxins have been categorized into three groups: (i) the small water-soluble solutes (MW < 500 Da), (ii) the middle molecules (MW > 500 Da) and (iii) the protein-bound uremic toxins (PBUTs, MW < 500 Da) [13,14].

Unlike water-soluble solutes, the removal of PBUTs is hampered during dialysis since only the free (*i.e.* unbound) fraction can pass the pores of the hemodialyzer membrane. Consequently, the removal efficiency of these PBUTs is much lower than of small water-soluble solutes, such as urea, especially for those with a high percentage protein binding (%PB) [15–17]. During the past decade, studies have been performed aiming to improve the clearance of these PBUTs during hemodialysis. Except for the studies on extended HD, most of them only focused on the removal of PBUTs from the serum/plasma compartment [18–21] ignoring the role of extra-plasmatic distribution [17,22].

To date, the kinetics (e.g. protein binding, distribution in the patient, clearance during hemodialysis) of PBUTs are not well understood (Figure 7-1). It is often hypothesized that the %PB of uremic toxins might be decreased in patients with CKD as compared to healthy subjects, because of the possible saturation of plasma proteins as a consequence of the elevated uremic toxin concentrations in these patients [23]. Additionally, it has been suggested that the %PB might be influenced by competition between different PBUTs bound to the same binding site on albumin [24–26], as well as between PBUTs and drug compounds [24,27–34]. Also, the distribution of PBUTs in the body is not yet completely characterized. So far, it has been found that PBUT transport in the patient is hampered during hemodialysis, suggesting the presence of these PBUTs not only in the patient's plasma volume, the only volume that is easily accessible during hemodialysis [17,22], but also in e.g. intracellular compartments. Consequently, the rate of transport across biological membranes could play an important role in the removal of these PBUTs during HD and with it, the removal efficiency. Assessing intracellular distribution by direct measurements is, however, limited to erythrocytes and leukocytes. Finally, the effects of laboratory and sample parameters on the free concentration of PBUTs (e.g. matrix, temperature, pH) are not clear but should be known since the %PB is usually calculated from the total and free PBUT concentration.

Therefore, the presented work was performed aiming to better understand the kinetics of PBUTs in CKD. Different processes which may play a role either in the determination of free PBUT concentrations (*i.e.* the matrix, temperature and pH) or in the kinetics (*i.e.* altered protein binding in CKD, protein binding competition between the different PBUTs and/or between drug compounds, the distribution in erythrocytes and the corresponding transport across the erythrocyte membrane) of these PBUTs were studied and are schematically presented in Figure 7-1. Studied PBUTs included *p*-cresyl glucuronide (*p*CG), hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS).

Figure 7-1 Research questions about the different processes impacting protein binding and kinetics of PBUTs.



HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate; CKD: chronic kidney disease; drug: vancomycin and teicoplanin.

The basic principles of the analytical methods which were used in this thesis are described in **Chapter 2**. In the presented work, PBUTs were quantified in serum or plasma samples using (ultra-)high performance liquid chromatography (UHPLC) methods where PBUTs were detected by ultraviolet and fluorescence detection (UHPLC-UV/FLD) or tandem mass spectrometry (UHPLC-MS/MS). The antibiotics vancomycin and teicoplanin were assayed by a chemiluminescent microparticle immunoassay (CMIA) and an UHPLC – high resolution mass spectrometry (UHPLC-HRMS) method, respectively. Furthermore, the importance of sample preparation in (bio)analytical chemistry was highlighted as well as the theory of the two most commonly used methods to obtain free concentrations, *i.e.* equilibrium dialysis and ultrafiltration.

In **Chapter 3**, the development and optimization of analytical methods which were used in this thesis are discussed. In general, the percentage protein binding (%PB) of a compound is indirectly determined by measuring the free and total concentration of that compound. Both equilibrium dialysis and ultrafiltration (Centrifree and Amicon filters) were used in this thesis and for this reason, we tested whether PBUTs were susceptible to non-specific adsorption to the membranes and/or devices. Only HA showed a small non-specific adsorption (sample loss of 11.5 %) to the equilibrium dialysis membrane and/or device but was not taken into account in further experiments, because it was supposed that the extent of non-specific adsorption is equal at both sides of the membrane. In this way, the potential error introduced by non-specific

adsorption is eliminated when measuring the concentrations at both sides to calculate the free fraction, which was the case for each study where equilibrium dialysis was used. We also compared the free PBUT concentrations as obtained by equilibrium dialysis and ultrafiltration (Amicon filters only) because this analysis was previously not performed, except for IS and *p*CS where Centrifree filters were used [35]. At the tested settings, we found comparable free concentrations, and thus %PB, for all four PBUTs when using equilibrium dialysis and ultrafiltration. Hence, both tested settings were used in the studies in this thesis.

Different methods have previously been developed to determine the %PB of PBUTs [35–37] but none of these described the impact on their %PB of different measurement conditions, except for temperature for IS and *p*CS in Viaene *et al.* (room temperature *versus* 37 °C) [35]. We demonstrated that the %PB of PBUTs could be assessed in both serum and K_2EDTA plasma when analyzed by UHPLC-UV/FLD and that the observed small difference in pH between serum and plasma samples had no influence on %PB. In addition, our results revealed the importance of ultrafiltration temperature when separating the free PBUT fraction. In addition, at 4 °C, %PB is significantly higher as compared to 37 °C or room temperature and is in line with data for drug compounds [38–40]. In order to reflect the physiologic situation, we recommend to perform ultrafiltration at 37 °C. This temperature-dependent relationship for %PB of PBUTs was previously only reported for IS and *p*CS, in which other ultrafiltration filters were used, but the same conclusions were made as in our work [35].

In the last part of **Chapter 3**, the development of a novel method for the quantification of total and free teicoplanin in K_2EDTA plasma samples by UHPLC-HRMS is provided. Also here, both equilibrium dialysis and ultrafiltration were tested for non-specific adsorption and obtained %PB values were compared. Non-specific adsorption was observed in two different ultrafiltration filters resulting in a mean sample loss of 77 and 21 % for Amicon and Centrifree UF filters, respectively. Despite the frequent use of ultrafiltration filters to obtain free teicoplanin concentrations [41–43], only one study previously reported a significant non-specific adsorption to ultrafiltration membranes and the authors introduced a correction factor for this [41]. Because of the lowest sample loss, we decided to retain Centrifree filters only and to filter calibration standards as well. We were the first to describe the good agreement in %PB of teicoplanin in spiked plasma between equilibrium dialysis and ultrafiltration at the used settings. Also here, the %PB assessed at 37 °C was compared to room temperature and we decided to use the 37 °C setting, as in Roberts *et al.* [43]. The apparent higher %PB of teicoplanin (around 97 %) reported in Dykhuizen *et al.* [41] might be caused by the UF

temperature of 4 °C. This temperature was not compared in the thesis, but in analogy to PBUTs and other protein-bound drugs, this low temperature may result in a higher %PB. In patient samples, a mean %PB of 87.7 % was demonstrated (range: 79.6 – 95.4 %) when assessed at 37 °C. This large inter-patient variability was previously reported by others as well [42,43]. Therefore, target attainment by monitoring the free (*i.e.* biologically active) concentration might be more suitable as compared to estimating free concentrations using a mean fraction of e.g. 10 %.

It should be noted that our developed and validated UHPLC-HRMS method is not the only one to quantify both total and free teicoplanin concentrations [41–43], but our approach is different from others by (i) the small plasma volume required for total and free teicoplanin quantification (*i.e.* 200 µL *versus* 700 µL in [43] and > 1 mL in [41]), (ii) the short analysis run time (*i.e.* 4.5 min *versus* 17 min in [43]), and (iii) the high specificity which was achieved by the high mass resolution of 70 000. Method comparison between the QMS® teicoplanin assay, which is used in routine laboratories for the quantification of total teicoplanin only, and our UHPLC-HRMS method revealed a moderate agreement with a correlation coefficient r = 0.82, which is in line with the correlation found for total teicoplanin by Mueller *et al.* [44]. The developed UHPLC-HRMS method can be useful in future therapeutic drug monitoring of teicoplanin, especially when the free concentration needs to be known. In this thesis, the newly developed method was used to compare the %PB of teicoplanin in a plasma pool of healthy subjects *versus* HD patients and to evaluate the potential competition with PBUTs (**Chapter 6**).

In **Chapter 4**, we reported the %PB of PBUTs in patients at different stages of CKD (*i.e.* stages 2 to 5) as well as in HD patients during a dialysis session in which the %PB was determined in samples taken at different time points. We attributed the observed decrease in %PB when normalized to the total concentration with decreasing creatinine clearance to protein binding competition amongst the different PBUTs; by the saturation of plasma proteins with PBUTs or protein-bound drugs; or by structural changes of albumin, caused by post-translational modifications. During an HD session, we observed an increase in %PB of PBUTs at the end of the session as well as after one passage through the hemodialyzer. A possible explanation for this could be the slow re-equilibration of the free fraction in the plasma and/or the slow input from the extra-plasmatic compartment [45], resulting in a continuously disturbed equilibrium in the hemodialyzer and even during the total dialysis session.

These *in vivo* observations were difficult to explain and only hypotheses could be made because of the limited information on the kinetics of PBUTs available in literature. Therefore, subsequent studies in this thesis were performed to better understand the kinetics of PBUTs in CKD.

In **Chapter 5**, the lower binding capacity of albumin in HD patients was demonstrated by deriving a binding curve for each of the studied PBUTs in a serum pool from healthy subjects as well as in a serum pool from HD patients (cleared and untreated). No difference in binding capacity of albumin was found between the serum pool of HD patients which was cleared from possible binding competitors as compared to untreated serum, except for HA. This was a first indication that competition for protein binding might be of minor importance for the studied PBUTs and was confirmed in the binding competition experiments where competition was only relevant at high uremic concentrations. The binding curves also demonstrated that albumin is not saturated with the studied PBUTs, even not at high uremic concentrations. Therefore, we proposed that the difference in protein binding between healthy and HD serum could at least in part be attributed to post-translational modifications of albumin, resulting in conformational changes and subsequent alterations in binding capacity. So far, only one paper demonstrated the direct impact of such a post-translational modification (*i.e.* guanidinylation) on the binding capacity of albumin for IS and tryptophan [46]. Thus, this field should be further explored in order to improve the knowledge of the impact of post-translational modifications on the binding capacity of albumin, as for example in the field of protein-bound drugs.

In the second part of **Chapter 5**, the %PB of two commonly used antibiotics in HD patients, *i.e.* vancomycin (%PB ~ 55 %) and teicoplanin (%PB ~ 95 %), was compared in a plasma pool from healthy subjects and in a plasma pool from HD patients. No difference in %PB of neither vancomycin nor teicoplanin was found between both pools, which may imply that at clinically relevant concentrations the albumin binding sites are not saturated in HD plasma.

For vancomycin, an *in vitro* %PB of around 60% was observed when both pools were spiked with a high concentration and this value was higher than previously reported *in vivo* [40,47,48]. It should be noted that the variability in reported %PB values for vancomycin is large within the same patient population but also between different patient populations [40,47,48]. In addition, differences in sample handling to obtain free vancomycin can also affect the observed %PB [39,40]. Because our aim was only to compare the %PB in plasma of healthy subjects and

HD patients, we did not take into account the potential inter-patient variability and used only two distinct plasma pools.

While no comparable studies were performed for teicoplanin, two independent studies were conducted for vancomycin [49,50]. In one of these studies, the %PB of vancomycin was compared between patients with normal renal function and patients with different renal functions [49], while for the other study, patients with end-stage kidney disease [50] were included. Both studies reported a lower mean %PB of vancomycin as compared to our study, *i.e.* 30 % and 18.5 % in [49] and [50], respectively. This discrepancy in %PB may potentially be explained by a difference between *in vivo* and *in vitro* experiments and/or by the large inter-patient variability [40,47,48]. Although a decreased %PB of vancomycin in patients with end-stage kidney disease as compared to patients with normal renal function was reported by Tan *et al.*, we could not confirm this in our *in vitro* experiment. However, our findings are more in line with other basic drug compounds which were previously studied in patients with renal failure. For these drugs, the %PB was found to be either increased (e.g. clonidine and propranolol [28]) or unchanged (e.g. penbutolol [51] and gentamicin [52]).

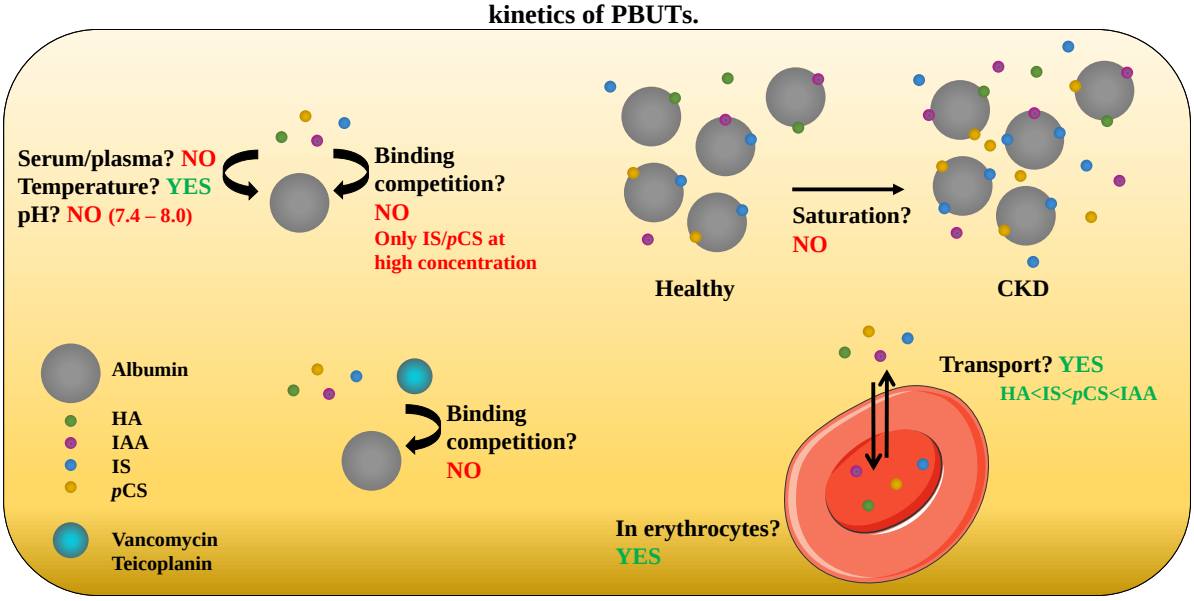
For teicoplanin, we observed no protein binding competition with PBUTs, while for vancomycin a small increase in %PB was registered. Changes in %PB of PBUTs might be of limited biological relevance so that it can be postulated that protein binding competition is absent. Possibly, these results may imply that vancomycin and teicoplanin bind to different binding sites on albumin than PBUTs and/or the number of binding sites on albumin are not saturated in the studied clinically relevant concentration ranges.

To address the possibility of extra-plasmatic distribution of PBUTs, we demonstrated in **Chapter 6** their presence in erythrocytes from HD patients and derived parameters describing the transport across the erythrocyte membrane. This was performed *in vitro* in a series of loading and unloading experiments of erythrocytes (in absence of plasma proteins) from healthy subjects and HD patients, respectively. For each PBUT, the intracellular partition was determined and was found to be higher than 1, which implies a higher concentration in the erythrocytes as compared to the plasma. This resulted in an apparent equilibrium and might be explained by the interaction of these toxins with membrane structures and/or cytoplasmic proteins. However, when we studied the possible binding of these PBUTs to hemoglobin in a small *in vitro* experiment, the PBUT-hemoglobin interaction seemed negligible. For each PBUT, we found that the rate of influx was comparable to the rate of efflux. Amongst the

different PBUTs, however, the rate of transport across the erythrocyte membrane increased according to the sequence HA < IS < *p*CS < IAA.

We also studied the impact of an inhibitor (*i.e.* 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid, DIDS) for anionic transport. This compound is known to bind reversibly to Band 3 proteins, the anion exchangers located on erythrocyte membranes [53–56]. For HA, IS and *p*CS, inhibiting Band 3 proteins resulted in a decrease in transport across the erythrocyte membrane, but did not stop it. For this reason, the transport of HA, IS and *p*CS may, at least partly, be attributed to the Band 3 protein and a second transport mechanism. From our experiments, however, we were not able to elucidate the exact transport mechanism for IAA nor for HA, IS and *p*CS, besides Band 3 transport.

Figure 7-2 Answers to the research questions about the different processes impacting protein binding and kinetics of PBUTs.



HA: hippuric acid; IAA: indole-3-acetic acid; IS: indoxyl sulfate; *p*CS: *p*-cresyl sulfate; CKD: chronic kidney disease; drug: vancomycin and teicoplanin.

7.2 Conclusion

In conclusion (Figure 7-2), to determine the %PB of PBUTs, a first step is to separate the free and bound fraction by either equilibrium dialysis or ultrafiltration and it is recommended to perform this at 37 °C. The free fraction, and thus the calculation of %PB, is independent (when measured by UHPLC-UV/FLD) of the sample matrix (*i.e.* serum or plasma), not influenced by a single freeze/thaw step, and is constant for a pH in the range 7.4 to 8.0. As compared to healthy subjects, %PB in uremia is lower for PBUTs (*in vivo* and *in vitro*) but unchanged for the studied antibiotics vancomycin and teicoplanin (*in vitro*). The %PB of only the highest

bound PBUTs is influenced by the presence of other highly bound PBUTs at high uremic concentrations, and not by the presence of lower bound PBUTs and/or vancomycin and teicoplanin, at least not *in vitro*. Hence, since competition cannot fully explain differences in %PB in uremic and healthy blood and since albumin binding sites are not saturated in uremic blood, it seems more obvious to attribute this difference in %PB to post-translational modifications of albumin. Finally, PBUT removal during dialysis seems also hampered by the uneven partition of PBUTs in the erythrocytes as well as by the different but still incompletely elucidated transport through the cell membrane according to the trend HA < IS < *p*CS < IAA.

7.3 Relevance of the work and future perspectives

Because of their protein binding, the removal of protein-bound uremic toxins (PBUTs) during hemodialysis (HD) is much lower as compared to small water-soluble solutes, especially for those with a high percentage protein binding (%PB). Since many of the PBUTs have been associated with, amongst others, inflammation and cardiovascular disease, it is of great interest to improve their removal during HD. To achieve this, the full picture of PBUT kinetics in the patient and the extracorporeal circuit needs to be understood.

To address this, the kinetics of PBUTs and two frequently administered protein-bound antibiotics were explored either *in vivo* and/or in different *in vitro* settings.

First, analytical techniques to determine the total and free concentrations of PBUTs as well as of the antibiotic teicoplanin were either optimized or developed, respectively. The pre-analytical step, *i.e.* the sample preparation, for total and free PBUT determination was further fine-tuned because of either inconsistencies or a lack of information in literature. The role of this sample preparation may not be underestimated because this may result in an under- or overestimation of the true *in vivo* values %PB. Because of the interest to measure free, *i.e.* biologically active and thus therapeutically relevant, concentrations of teicoplanin, and the large inter-patient (and even intra-patient) variability in the free fraction, no general rule-of-thumb can be introduced to estimate the free teicoplanin concentration. For this reason, a novel ultra-high performance liquid chromatography method with high resolution mass spectrometry was developed and validated. A drawback of our new method is, however, that individual measurements of total concentrations deviated up to 50 % between our method and the QMS teicoplanin method, which is used for routine analyses. This is clinically not acceptable, and further research would be recommended to unravel this discrepancy. In addition, it could be interesting to investigate the origin of the interfering compound for teicoplanin A2-4 & A2-5.

Second, we demonstrated that the findings of our *in vivo* study, in which the %PB of PBUTs decreased with decreasing renal function, were not attributed to protein binding competition amongst different PBUTs, nor by protein binding competition with two antibiotics (*i.e.* vancomycin and teicoplanin), at least not *in vitro*. In addition, this decrease in %PB could not be attributed to the saturation of albumin either. These novel insights in the binding characteristics of PBUTs may implicate that the difference in %PB of PBUTs (and probably for some drug compounds as well) in uremic and healthy blood is more likely to be caused by

post-translational modifications of albumin. With this new information, it became clear that albumin has a high binding capacity, which is decreased in HD patients, but at uremic levels, albumin is still capable to bind many ligands. Future studies can be performed to further unravel the magnitude and effect of post-translational modifications on the %PB of PBUTs and of different drug molecules.

Since the two antibiotics vancomycin and teicoplanin are mainly excreted by the kidneys and most patients on HD have no or only a small residual renal function, their elimination in HD patients is largely dependent on the clearance of the dialyzer. In addition, it is often hypothesized that the free fraction of drugs is changed in uremia as compared to healthy blood, as a result of binding competition with PBUTs and/or of albumin saturation, but without (much) data supporting this. The dosage regimen of vancomycin and teicoplanin, as well as of other antibiotics, in HD patients is often estimated based on the residual renal function, but not on the possible higher or lower free fraction, while it is the free concentrations that is responsible for the therapeutic action. When the free fraction, and thus %PB, is changed in uremia as compared to patients with normal renal function and the dosage is not adapted to this change, this could result in under- or overdoses and thus a higher risk for resistance against the antibiotic or a higher risk for toxicity, respectively. Therefore, we compared the %PB of vancomycin and teicoplanin between healthy and uremic blood, in an *in vitro* setting and found no difference, neither relevant competition with PBUTs. In a next step, the %PB of these (and other) antibiotics should be compared *in vivo* to incorporate intra- and inter-patient variability and to see if our *in vitro* findings can be translated to *in vivo* data. Furthermore, including more protein-bound drugs could potentially help to interpolate for other drugs from the same category. Ultimately, the improved knowledge on the kinetics of antibiotics in HD patients could help to fine tune dosing recommendations.

Third, as already mentioned in the general discussion, several studies have previously been performed aiming to improve the clearance of PBUTs during hemodialysis, but only focused on the removal from the serum/plasma compartment by e.g. increasing the free fraction or by combining dialysis with adsorption, ignoring their possible extra-plasmatic distribution. In this thesis, we demonstrated the presence of PBUTs in an easily accessible compartment, *i.e.* the erythrocytes. Their transport across erythrocyte membranes was studied and kinetic parameters were derived to further fine tune kinetic modeling of these PBUTs during dialysis. For this purpose, diffusion-adjusted regional blood flow models offer a higher physiologic relevance as compared to standard serial two-compartment models, because they differentiate between

systems with a low (e.g. muscle and skin) and a high (e.g. liver and kidney) blood flow and take into account the transport across intra- and extracellular compartments. Future experiments, e.g. adding albumin to test samples, adding DIDS when loading erythrocytes from HD patients etc. should be conducted to describe the transport of PBUTs in more physiologically relevant conditions. Eventually, this may help to further improve the removal of PBUTs in patients treated with HD, possibly by adapting removal strategies, and finally to result in a better patient outcome and survival.

7.4 References

1. Levey AS, Coresh J, Bolton K, Culleton B, Harvey KS, Ikizler AT (2002) K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am. J. Kidney Dis. 39:S1-266

2. Schepers E, Meert N, Glorieux G, Goeman J, Van der Eycken J, Vanholder R (2007) P-cresylsulphate, the main in vivo metabolite of p-cresol, activates leucocyte free radical production. Nephrol Dial Transplant 22:592–596. doi: 10.1093/ndt/gfl584

3. Adijiang A, Goto S, Uramoto S, Nishijima F, Niwa T (2008) Indoxyl sulphate promotes aortic calcification with expression of osteoblast-specific proteins in hypertensive rats. Nephrol Dial Transplant 23:1892–901. doi: 10.1093/ndt/gfm861

4. Barreto FC, Barreto D V, Liabeuf S, Meert N, Glorieux G, Temmar M, Choukroun G, Vanholder R, Massy ZA (2009) Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. Clin J Am Soc Nephrol 4:1551–1558. doi: 10.2215/CJN.03980609

5. Meijers BKI, Van kerckhoven S, Verbeke K, Dehaen W, Vanrenterghem Y, Hoylaerts MF, Evenepoel P (2009) The Uremic Retention Solute p-Cresyl Sulfate and Markers of Endothelial Damage. Am J Kidney Dis 54:891–901. doi: 10.1053/j.ajkd.2009.04.022

6. Liabeuf S, Barreto D V., Barreto FC, Meert N, Glorieux G, Schepers E, Temmar M, Choukroun G, Vanholder R, Massy Z a. (2010) Free p-cresylsulphate is a predictor of mortality in patients at different stages of chronic kidney disease. Nephrol Dial Transplant 25:1183–1191. doi: 10.1093/ndt/gfp592

7. Jourde-Chiche N, Dou L, Cerini C, Dignat-George F, Brunet P (2011) Vascular Incompetence in Dialysis Patients-Protein-Bound Uremic Toxins and Endothelial Dysfunction. Semin Dial 24:327–337. doi: 10.1111/j.1525-139X.2011.00925.x

8. Sirich TL, Aronov PA, Plummer NS, Hostetter TH, Meyer TW (2013) Numerous protein-bound solutes are cleared by the kidney with high efficiency. Kidney Int 84:585–90. doi: 10.1038/ki.2013.154

9. Vanholder R, Schepers E, Pletinck A, Nagler E, Glorieux G (2014) The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review. J Am Soc Nephrol 1–11.

doi: 10.1681/ASN.2013101062

10. Vanholder R, Pletinck A, Schepers E, Glorieux G (2018) Biochemical and Clinical Impact of Organic Uremic Retention Solutes: A Comprehensive Update. Toxins (Basel) 10:33. doi: 10.3390/toxins10010033

11. Vanholder R, Massy Z, Argiles A, Spasovski G, Verbeke F, Lameire N (2005) Chronic kidney disease as cause of cardiovascular morbidity and mortality. Nephrol Dial Transplant 20:1048–1056. doi: 10.1093/ndt/gfh813

12. Matsushita K, van der Velde M, Astor B, Woodward M, Levey A, de Jong P, Coresh J, Gansevoort R (2010) Association of estimated glomerular fi ltration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. www.thelancet.com Lancet 375:2073–81. doi: 10.1016/S0140-6736(10)60674-5

13. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clarck W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jörres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C, Zidek W (2003) Review on uremic toxins: Classification, concentration and interindividual variability. Am J Soc Nephrol 63:1934–1943. doi: 10.1046/j.1523-1755.2003.00924.x

14. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A (2012) Normal and Pathologic Concentrations of Uremic Toxins. J Am Soc Nephrol 23:1258–1270. doi: 10.1681/ASN.2011121175

15. Vanholder R, De Smet R, Lesaffer G (2002) Dissociation between dialysis adequacy and Kt/V. Semin Dial 15:3–7. doi: 10.1046/j.1525-139x.2002.00005.x

16. Eloot S, Van Biesen W, Dhondt A, Van de Wynkele H, Glorieux G, Verdonck P, Vanholder R (2008) Impact of hemodialysis duration on the removal of uremic retention solutes. Kidney Int 73:765–770. doi: 10.1038/sj.ki.5002750

17. Eloot S, Schneditz D, Cornelis T, Van Biesen W, Glorieux G, Dhondt A, Kooman J, Vanholder R (2016) Protein-bound uremic toxin profiling as a tool to optimize hemodialysis. PLoS One 11:1–18. doi: 10.1371/journal.pone.0147159

18. Meert N, Eloot S, Schepers E, Lemke H-D, Dhondt A, Glorieux G, Landschoot M Van,

Waterloos M-A, Vanholder R (2011) Comparison of removal capacity of two consecutive generations of high-flux dialysers during different treatment modalities. Nephrol Dial Transpl 26:2624–2630. doi: 10.1093/ndt/gfq803

19. Cornelis T, Eloot S, Vanholder R, Glorieux G, Van Der Sande FM, Scheijen JL, Leunissen KM, Kooman JP, Schalkwijk CG (2015) Protein-bound uraemic toxins, dicarbonyl stress and advanced glycation end products in conventional and extended haemodialysis and haemodiafiltration. Nephrol Dial Transplant 30:1395–1402. doi: 10.1093/ndt/gfv038

20. Böhringer F, Jankowski V, Gajjala PR, Zidek W, Jankowski J (2015) Release of Uremic Retention Solutes from Protein Binding by Hypertonic Predilution Hemodiafiltration. ASAIO J 61:55–60. doi: 10.1097/MAT.0000000000000166

21. Pavlenko D, van Geffen E, van Steenbergen MJ, Glorieux G, Vanholder R, Gerritsen KGF, Stamatialis D (2016) New low-flux mixed matrix membranes that offer superior removal of protein-bound toxins from human plasma. Sci Rep 6:34429. doi: 10.1038/srep34429

22. Maheshwari V, Thijssen S, Tao X, Fuertinger D, Kappel F, Kotanko P (2017) A novel mathematical model of protein-bound uremic toxin kinetics during hemodialysis. Sci Rep 7:10371. doi: 10.1038/s41598-017-10981-z

23. Klammt S, Wojak HJ, Mitzner A, Koball S, Rychly J, Reisinger EC, Mitzner S (2012) Albumin-binding capacity (ABiC) is reduced in patients with chronic kidney disease along with an accumulation of protein-bound uraemic toxins. Nephrol Dial Transplant 27:2377–2383. doi: 10.1093/ndt/gfr616

24. Bertuzzi A, Mingrone G, Gandolfi A, Greco A V., Ringoir S, Vanholder R (1997) Binding of indole-3-acetic acid to human serum albumin and competition with L-tryptophan. Clin Chim Acta 265:183–192.

25. Mingrone G, De Smet R, Greco AV, Bertuzzi A, Gandolfi A, Ringoir S, Vanholder R (1997) Serum uremic toxins from patients with chronic renal failure displace the binding of L-tryptophan to human serum albumin. Clin Chim Acta 260:27–34. doi: 10.1016/S0009-8981(96)06504-7

26. Bergé-Lefranc D, Chaspoul F, Cérini C, Brunet P, Gallice P (2013) Thermodynamic

study of indoxylsulfate interaction with human serum albumin and competitive binding with p-cresylsulfate. J Therm Anal Calorim 115:2021–2026. doi: 10.1007/s10973-013-3067-6

27. Perucca E (1980) Plasma Protein Binding of Phenytoin in Health and Disease: Relevance to Therapeutic Drug Monitoring. Ther Drug Monit 2:331–344.

28. Vanholder R, Van Landschoot N, De Smet R, Schoots A, Ringoir S (1988) Drug protein binding in chronic renal failure: evaluation of nine drugs. Kidney Int 33:996–1004. doi: 10.1038/ki.1988.99

29. Mabuchi H, Nakahashi H (1988) Displacement by Anionic Drugs of Endogenous Ligands Bound to Albumin in Uremic Serum. Ther Drug Monit 10:261–264.

30. Takamura N, Maruyama T, Otagiri M (1997) Effects of uremic toxins and fatty acids on serum protein binding of furosemide: Possible mechanism of the binding defect in uremia. Clin Chem 43:2274–2280.

31. Davilas A, Koupparis M, Macheras P, Valsami G (2006) In-vitro study on the competitive binding of diflunisal and uraemic toxins to serum albumin and human plasma using a potentiometric ion-probe technique. J Pharm Pharmacol 58:1467–1474. doi: 10.1211/jpp.58.11.0007

32. Johannessen Landmark C, Johannessen SI, Tomson T (2012) Host factors affecting antiepileptic drug delivery-Pharmacokinetic variability. Adv Drug Deliv Rev 64:896–910. doi: 10.1016/j.addr.2011.10.003

33. Zaidi N, Ahmad E, Rehan M, Rabbani G, Ajmal MR, Zaidi Y, Subbarao N, Khan RH (2013) Biophysical Insight into Furosemide Binding to Human Serum Albumin : A Study To Unveil Its Impaired Albumin Binding in Uremia. doi: 10.1021/jp3069877

34. Tao X, Thijssen S, Kotanko P, Ho C, Henrie M, Stroup E, Handelman G (2016) Improved dialytic removal of protein-bound uraemic toxins with use of albumin binding competitors : an in vitro human whole blood study. Nat Publ Gr 2–10. doi: 10.1038/srep23389

35. Viaene L, Annaert P, De Loor H, Poesen R, Evenepoel P, Meijers B (2013) Albumin is the main plasma binding protein for indoxyl sulfate and p-cresyl sulfate. Biopharm Drug Dispos 34:165–175. doi: 10.1002/bdd.1834

36. Fagugli RM, De Smet R, Buoncristiani U, Lameire N, Vanholder R (2002) Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. Am J Kidney Dis 40:339–347. doi: 10.1053/ajkd.2002.34518

37. Itoh Y, Ezawa A, Kikuchi K, Tsuruta Y, Niwa T (2012) Protein-bound uremic toxins in hemodialysis patients measured by liquid chromatography/tandem mass spectrometry and their effects on endothelial ROS production. Anal Bioanal Chem 403:1841–1850. doi: 10.1007/s00216-012-5929-3

38. Nilsson LB (2013) The bioanalytical challenge of determining unbound concentration and protein binding for drugs. Bioanalysis 5:3033–50. doi: 10.4155/bio.13.274

39. Kratzer A, Liebchen U, Schleibinger M, Kees MG, Kees F (2014) Determination of free vancomycin, ceftriaxone, cefazolin and ertapenem in plasma by ultrafiltration: Impact of experimental conditions. J Chromatogr B Anal Technol Biomed Life Sci 961:97–102. doi: 10.1016/j.jchromb.2014.05.021

40. Stove V, Coene L, Carlier M, Waele JJ De, Fiers T, Verstraete AG (2015) Measuring Unbound Versus Total Vancomycin Concentrations in Serum and Plasma : Methodological Issues and Relevance. Ther Drug Monit 37:180–187.

41. Dykhuizen RS, Harvey G, Stephenson N, Nathwani D, Gould IM (1995) Protein binding and serum bactericidal activities of vancomycin and teicoplanin. Antimicrob Agents Chemother 39:1842–1847. doi: 10.1128/AAC.39.8.1842

42. Yano R, Nakamura T, Tsukamoto H, Igarashi T, Goto N, Wakiya Y, Masada M (2007) Variability in teicoplanin protein binding and its prediction using serum albumin concentrations. Ther Drug Monit 29:399–403. doi: 10.1097/FTD.0b013e3180690755

43. Roberts JA, Stove V, De Waele JJ, Sipinkoski B, McWhinney B, Ungerer JPJ, Akova M, Bassetti M, Dimopoulos G, Kaukonen KM, Koulenti D, Martin C, Montravers P, Rello J, Rhodes A, Starr T, Wallis SC, Lipman J (2014) Variability in protein binding of teicoplanin and achievement of therapeutic drug monitoring targets in critically ill patients: Lessons from the DALI Study. Int J Antimicrob Agents 43:423–430. doi: 10.1016/j.ijantimicag.2014.01.023

44. Mueller DM, Von Eckardstein A, Saleh L (2014) Quantification of teicoplanin in plasma by LC-MS with online sample clean-up and comparison with QMS (R) assay. Clin Chem

Lab Med 52:879–887. doi: 10.1515/cclm-2013-0974

45. Eloot S, Vanholder R (2012) Kinetics of protein-bound solutes during hemodialysis. Int J Artif Organs 35:583.

46. Rueth M, Lemke H-D, Preisinger C, Krieter D, Theelen W, Gajjala P, Devine E, Zidek W, Jankowski J, Jankowski V (2015) Guanidinylations of albumin decreased binding capacity of hydrophobic metabolites. Acta Physiol 215:13–23. doi: 10.1111/apha.12518

47. Butterfield JM, Patel N, Pai MP, Rosano TG, Drusano GL, Lodise TP (2011) Refining vancomycin protein binding estimates: identification of clinical factors that influence protein binding. Antimicrob Agents Chemother 55:4277–82. doi: 10.1128/AAC.01674-10

48. Oyaert M, Spriet I, Allegaert K, Smits A, Vanstraelen K, Peersman N, Wauters J, Verhaegen J, Vermeersch P, Pauwels S (2015) Factors impacting unbound vancomycin concentrations in different patient populations. Antimicrob Agents Chemother 59:7073–9. doi: 10.1128/AAC.01185-15

49. Rodvold KA, Blum RA, Fischer JH, Zokufa HZ, Rotschafer JC, Crossley KB, Rifes LJ, Golper A, Elzinga L, Noonan H, Anderson J, Gilbert DN, Bennett WM (1988) Vancomycin Pharmacokinetics in Patients with Various Degrees of Renal Function. Antimicrob AGENTS Chemother Conf Antimicrob Agents Chemother 848–852.

50. Tan CC, Lee HS, Ti TY, Lee EJC (1990) Pharmacokinetic of Intravenous Vancomycin in Patients with End-Stage Renal Failure. Ther Drug Monit 12:29–34.

51. Aguirre C, Calvo R, Rodriguez-Sasiain JM (1993) Unchanged protein binding of penbutolol in renal insufficiency : a possible role of carbamylation. Int J Clin Pharmacol 31:31–34.

52. Myers DR, DeFehr J, Bennet WM, Porter GA, Olsen GD (1978) Gentamicin binding to serum and plasma proteins. Clin Pharmacol Ther 23:356–60.

53. Soszynski M, Bartosz G (1997) Penetration of erythrocyte membrane by peroxynitrite: participation of the anion exchange protein. Biochem Mol Biol Int 43:319–325.

54. Salhany JM (2001) Stilbenedisulfonate binding kinetics to band 3 (AE 1): Relationship between transport and stilbenedisulfonate binding sites and role of subunit interactions in transport. Blood Cells, Mol Dis 27:127–134. doi: 10.1006/bcmd.2000.0369

55. Salhany JM, Schopfer LM (2001) Kinetic mechanism of DIDS binding to band 3 (AE1) in human erythrocyte membranes. Blood Cells, Mol Dis 27:844–849. doi: 10.1006/bcmd.2001.0458

56. Reithmeier RAF, Casey JR, Kalli AC, Sansom MSP, Alguel Y, Iwata S (2016) Band 3, the human red cell chloride/bicarbonate anion exchanger (AE1, SLC4A1), in a structural context. Biochim Biophys Acta 1858:1507–1532. doi: 10.1016/j.bbamem.2016.03.030.

Abstract

Background. As a result of decreased renal function, numerous solutes are accumulated in patients with chronic kidney disease (CKD). Many of these uremic retention solutes exert toxicity and have been classified into three groups, including small water soluble solutes, middle molecules and protein-bound uremic toxins (PBUTs). The percentage of solute bound to plasma proteins (percentage protein binding, %PB) varies amongst the different PBUTs and comprises the full range from almost 0 up to 100%. Because of this protein binding, removal efficiency of PBUTs during hemodialysis (HD) is much lower as compared to small water-soluble solutes, especially for those with a high %PB. Since many of the PBUTs have an impact on the cardiovascular system and contribute to the increased propensity for cardiovascular events and mortality in patients with CKD, it is of great interest to improve their removal during HD. For this, we need to understand the full picture of PBUT kinetics in the patient and the extracorporeal circuit.

Aims. The kinetics of PBUTs and protein-bound antibiotics were explored either *in vivo* and/or in different *in vitro* settings after development and/or optimization of analytical techniques.

Methods. Studied protein-bound solutes included (i) the uremic toxins *p*-cresyl glucuronide (*p*CG; only in the *in vivo* study), hippuric acid (HA), indole-3-acetic acid (IAA), indoxyl sulfate (IS) and *p*-cresyl sulfate (*p*CS), as ranked here for increasing %PB, and (ii) the frequently administered antibiotics vancomycin and teicoplanin.

The *in house* developed method for PBUT quantification was fine-tuned by checking the effect of sample temperature (*i.e.* 37 °C, room temperature and 4 °C), pH, matrix (*i.e.* serum and plasma) and a single freeze/thaw cycle on the %PB using blood samples from 10 HD patients. For the quantification of the antibiotic teicoplanin, a novel ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS) assay was developed and validated.

In an *in vivo* study, PBUT %PB data were collected in 95 patients with different stages of CKD and in 10 patients on HD. In these HD patients, kinetics with %PB changes were evaluated by blood sampling at different time points during an HD session from the arterial as well as venous blood line.

To better explain the *in vivo* results, binding characteristics of PBUTs and binding competition amongst them were explored *in vitro*. This was performed in a serum pool from healthy subjects as well as from HD patients (cleared and untreated) to include the possible impact of changes in albumin. In addition, %PB of the antibiotics was compared *in vitro* in a plasma pool from healthy subjects as well as from HD patients, and the possible binding competition with PBUTs was checked.

Finally, to better understand PBUT kinetics, their distribution in a direct accessible extra-plasmatic compartment, *i.e.* the erythrocytes, was assessed in blood from 6 HD patients and transport parameters were derived by loading erythrocytes of 8 healthy subjects with a mixture of PBUTs and by unloading erythrocytes of 8 HD patients.

Results. PBUT %PB was not influenced by the choice of matrix, sample pH and a single freeze/thaw cycle, while it increased with decreasing temperatures, such that the assessment of %PB should preferably be performed at 37 °C to mimic clinical conditions.

The novel highly sensitive UHPLC-HRMS method allowed accurate and precise quantification of free and total teicoplanin, and thus the %PB, in a total analysis run time of 4.5 min and required only 200 µL of plasma volume.

In CKD patients, the *in vivo* study revealed a decrease in PBUT %PB with creatinine clearance, when normalized to total PBUT concentration. During an HD session, %PB was increased at the dialysis end for the stronger (IAA) and the highly-bound (IS and *p*CS) PBUTs. In addition, %PB of these stronger and highly-bound PBUTs was found to increase when blood is passing through the hemodialyzer at 120 min after dialysis start.

We found that albumin has a higher binding capacity for PBUTs in healthy serum as compared to blank and non-treated HD serum. It was also observed that the number of bound PBUTs per albumin molecule increases linearly with free PBUT concentration even beyond high uremic levels. Binding competition was found between the two highly-bound PBUTs IS and *p*CS at high uremic concentrations. For the studied antibiotics, %PB was not different in healthy and uremic plasma as measured *in vitro*, and no biologically relevant changes in the %PB of PBUTs were observed in the presence of antibiotics.

PBUTs were found being distributed in erythrocytes, with an uneven partition between the intra- and extra-cellular compartment. The influx and efflux rate for transport across the

erythrocyte membrane was comparable for each PBUT, but increased according to the trend HA < IS < *p*CS < IAA.

Conclusion. The %PB of PBUTs should be determined at 37 °C, is independent of the sample matrix, not influenced by a single freeze/thaw step, and is constant for a pH in the range 7.4 to 8.0. As compared to healthy subjects, %PB in uremia is lower for PBUTs (*in vivo* and *in vitro*) but unchanged for the studied antibiotics vancomycin and teicoplanin (*in vitro*). Neither binding competition, nor albumin saturation can completely explain differences in %PB in uremic and healthy blood. It is more obvious to attribute this difference in %PB at least in part to post-translational modifications of albumin. Finally, PBUT removal during dialysis seems also hampered by the uneven partition of PBUTs in the erythrocytes as well as by the different but still incompletely elucidated transport through the cell membrane according to the trend HA < IS < *p*CS < IAA.

Samenvatting

Achtergrond. Patiënten met chronisch nierlijden stapelen een groot aantal componenten op in hun lichaam ten gevolge van de verlaagde nierfunctie. Veel van deze opgestapelde componenten, ook wel uremische retentiestoffen genoemd, hebben een toxisch effect en worden dan verdeeld in drie groepen, zijnde de kleine water oplosbare componenten, de middelgrote moleculen en de eiwitgebonden uremische toxines (protein-bound uremic toxins, PBUTs). Het percentage van een bepaalde uremische toxine dat aan plasma eiwitten gebonden is (percentage proteïne binding, %PB) is verschillend tussen de PBUTs en kan variëren van bijna 0 tot 100 %. Vanwege hun eiwitbinding worden PBUTs tijdens hemodialyse minder efficiënt verwijderd dan kleine water oplosbare componenten, voornamelijk als hun %PB hoog is. Veel van de PBUTs hebben een invloed op het cardiovasculair systeem en verhogen dus de kans op cardiovasculaire aandoeningen en mortaliteit in patiënten met chronisch nierlijden. Het is dus van groot belang om de verwijdering van deze PBUTs tijdens hemodialyse (HD) te verbeteren. Hiervoor moeten we echter de volledige kinetiek van de PBUTs begrijpen zowel in de patiënt zelf als in het extracorporale circuit.

Doel. De kinetiek van eiwitgebonden uremische toxines en eiwitgebonden antibiotica werd bestudeerd *in vivo* en/of in verschillende *in vitro* opstellingen na het ontwikkelen en/of optimaliseren van analytische technieken.

Methodes. Volgende eiwitgebonden componenten werden bestudeerd: (i) de PBUTs *p*-cresyl glucuronide (*p*CG; enkel in de *in vivo* studie), hippuurzuur (HA), indool-3-azijnzuur (IAA), indoxyl sulfaat (IS) en *p*-cresyl sulfaat (*p*CS), dewelke hier gerangschikt staan volgens een toenemende %PB en (ii) de regelmatig toegediende antibiotica vancomycine en teicoplanine.

De *in huis* ontwikkelde analyse methode voor de bepaling van PBUTs werd verfijnd door het effect van staaltemperatuur (d.i. 37 °C, kamertemperatuur en 4 °C), pH, matrix (d.i. serum of plasma) en één vries/dooi cyclus op het %PB na te gaan, door gebruik te maken van bloedstalen van 10 HD patiënten. Voor de bepaling van het antibioticum teicoplanine werd er een nieuwe ultra-hoge performante vloeistofchromatografie – hoge resolutie massaspectrometrie (UHPLC-HRMS) methode ontwikkeld en gevalideerd.

Het %PB van PBUTs werd berekend in een *in vivo* studie bij 95 patiënten met verschillende stadia van chronisch nierlijden en bij 10 patiënten die behandeld werden met HD. Verandering

in %PB van PBUTs werd geëvalueerd in HD patiënten door bloedstalen te verzamelen van de arteriële en veneuze bloedlijnen op verschillende tijdspunten tijdens een dialysesessie.

Om de resultaten van de *in vivo* studie beter te kunnen begrijpen werden de bindingskarakteristieken en de onderlinge bindingscompetitie van PBUTs verkend. Dit werd nagegaan *in vitro* in een serummengsel van gezonde vrijwilligers enerzijds en van HD patiënten (blanco en onbehandeld) anderzijds om mogelijke verschillen in albumine in rekening te brengen. Ook het %PB van twee antibiotica, vancomycine en teicoplanine, werd vergeleken *in vitro* in een plasmamengsel van gezonde vrijwilligers enerzijds en van HD patiënten anderzijds en de mogelijke bindingscompetitie met PBUTs werd bekeken.

Verder werd ook de verdeling van PBUTs bestudeerd in een eenvoudig toegankelijk extra-plasmatisch compartiment, d.i. de erythrocyten, in bloed van 6 HD patiënten. Transport parameters werden afgeleid via het laden van erythrocyten van 8 gezonde vrijwilligers met een mengsel van de toxines, alsook via het ontladen van erythrocyten van 8 HD patiënten.

Resultaten. Het %PB van PBUTs was onafhankelijk van de matrix, de pH van het staal en één vries/dooi cyclus, terwijl het wel steeg bij afnemende temperaturen. Hierdoor is het aangeraden het %PB te bepalen bij 37 °C om de klinische condities na te bootsen.

De nieuwe UHPLC-HRMS methode maakte het mogelijk om totaal en vrij teicoplanine, en dus het %PB, te kwantificeren op een zeer gevoelige, accurate en precieze manier, in een totale analysetijd van 4.5 min en het benodigd plasma volume bedroeg slechts 200 µL.

De *in vivo* studie toonde aan dat het %PB van eiwitgebonden uremische toxines, genormaliseerd voor de totale concentratie, daalde met afnemende renale functie in patiënten met chronisch nierlijden. Tijdens een dialysesessie steeg het %PB naar het einde toe voor de hoger gebonden (IAA) en de nog hoger gebonden (IS en *p*CS) toxines. Bovendien was het %PB van IAA, IS en *p*CS toegenomen als bloed doorheen de kunstnier stroomde, op 120 min na de start van de dialyse.

Het bleek dat albumine een hogere bindingscapaciteit heeft voor eiwitgebonden uremische toxines in gezond serum dan in HD serum (blanco en onbehandeld). Daarnaast steeg het absolute aantal toxines die gebonden is per albumine molecule lineair met de vrije toxine concentratie, tot voorbij hoge uremische concentraties. Bindingscompetitie tussen de verschillende PBUTs werd enkel vastgesteld bij hoge uremische concentraties van de hoog gebonden toxines IS en *p*CS. Voor vancomycine en teicoplanine werd er *in vitro* geen

significant verschil in %PB waargenomen tussen gezond en uremisch plasma, net zoals er geen biologisch relevante verandering in %PB van PBUTs in de aanwezigheid van de antibiotica werd vastgesteld.

Als laatste werd er gevonden dat PBUTs zich in erythrocyten verdelen, maar ook dat er een oneven verdeling is tussen het intra- en extracellulair compartiment. De influx en efflux snelheid voor transport doorheen de erythrocyt membraan was vergelijkbaar per PBUT, maar nam toe volgens de trend HA < IS < *p*CS < IAA.

Conclusie. De %PB van PBUTs wordt best bepaald op 37 °C, is onafhankelijk van de matrix, niet beïnvloed door één vries/dooi cyclus en is constant in een pH gebied van 7.4 tot 8.0. De %PB in uremie is lager voor de PBUTs (*in vivo* en *in vitro*) in vergelijking met gezonde personen, maar lijkt ongewijzigd voor de bestudeerde antibiotica vancomycine en teicoplanine (*in vitro*). Noch bindingscompetitie, noch albumine verzadiging kunnen de verschillen in %PB tussen uremisch en gezond blood volledig verklaren. Het is meer aannemelijk dat dit verschil in %PB, of toch gedeeltelijk, toegeschreven wordt aan posttranslationele modificaties van albumine. Als laatste kan gesteld worden dat de moeilijke verwijdering van PBUTs tijdens dialyse deels beïnvloed wordt door hun verdeling in extra-plasmatische compartimenten zoals de erythrocyten en daarbij hun verschillend, maar nog steeds onvolledig uitgeklaarde, transport doorheen de erythrocyt celmembraan, volgens de trend HA < IS < *p*CS < IAA.

Dankwoord

Na het afstuderen als Master of Science, Chemistry kreeg ik de opportuniteit om een doctoraat te starten op de dienst Nefrologie van het UZ Gent. Hoewel het ergens een stap in het ongewisse was, heb ik deze kans met beide handen genomen en heb ik de wereld van de geneeskunde en gezondheidswetenschappen kunnen verkennen. Tijdens dit vierjarig project heb ik het genoegen gehad te mogen samenwerken met verschillende onderzoekers uit verschillende diensten en/of universiteiten, met deze thesis als finaal resultaat. Graag wil ik alle personen bedanken die zowel rechtstreeks als onrechtstreeks bijgedragen hebben tot het welslagen van dit doctoraat.

Vooreerst wil ik mijn promotor, *Prof. dr. Sunny Eloot,* bedanken om mij te betrekken in dit project alsook om steeds tijd te maken om samen mijn experimenten en teksten te overlopen. Bedankt om me te begeleiden en te laten groeien tijdens mijn doctoraat, mijn schrijfvaardigheden te verbeteren en mijn kritische geest aan te scherpen. Onze verschillende achtergronden maakten het niet altijd eenvoudig, maar misschien heeft net dát geleid tot het slagen van dit doctoraat.

Prof. dr. Annemieke Dhondt, wil ik bedanken voor de inclusie van dialysepatiënten en voor het nalezen van verschillende werken.

Het fonds wetenschappelijk onderzoek (FWO) bedank ik voor de financiële steun via het project nummer G0A4614N.

Een speciaal woord van dank gaat naar *prof. dr. Griet Glorieux* die voor mij als een derde promotor was. Jij hielp me steeds bij het opstellen van mijn protocollen, tijdens het experimenteel deel en bij de interpretatie van de resultaten, wat zeker niet altijd evident was. Bedankt dat ik steeds bij jou terecht kon met mijn talloze vragen, alsook om mij mee te nemen naar de verschillende EUTox bijeenkomsten. Succes met de verdere plannen voor het nieuw onderzoek gebouw.

Prof. dr. Frédéric Lynen wil ik bedanken om mij als geschikte kandidaat voor dit project aan Sunny voor te stellen. Dit was zeker een evenwaardig alternatief voor het oorspronkelijk IWT project die we eerder samen ingediend hadden. Ook bedankt om me in het begin van mijn doctoraat te laten meedraaien binnen jouw onderzoeksgroep en mij te laten deelnemen aan de groepsactiviteiten.

Ook een dank je aan de laboranten van de dienst Nefrologie. Zonder jullie was ik niet in de mogelijkheid om zoveel experimenten uit te voeren en mijn thesis af te krijgen binnen de voorziene tijd. *Tom*, bedankt om het mannen aandeel in het labo te versterken en me o.a. te helpen bij de methodevalidatie van teicoplanine. Al weet ik dat ik je niet kon straffen om je naar het labo toxicologie te sturen ;-) *Mieke*, bedankt voor je hulp bij mijn vele experimenten en om af en toe iets op te zoeken, soms van vele jaren terug. *Sophie en Marie-Anne*, dank je om regelmatig bij te springen. Elk van jullie verdient alvast een dikke pluim voor inzet en nauwgezet werken. Ik laat jullie graag mijn sanseveria achter, maar geef haar toch één keer per week water :-)

Eva en Anneleen, merci voor de leuke babbels en om met een andere insteek mijn werk te beoordelen tijdens de vergaderingen. Ook bedankt om enkele hoofdstukken van mijn thesis na te lezen. Succes met jullie verder onderzoek.

Verder wil ik nog een woord van dank richten aan al het personeel van de dienst nefrologie. *Chantal* en *Christel*, bedankt voor de regeling van afspraken, bestellingen en financiën. De studie verpleegkundigen *Elsie, Kelly, Isabelle, Petra* en *Cathy* wil ik bedanken voor de vele bloedafnames die nodig waren voor mijn experimenten alsook voor de gezellige lunches. Ook een dank je aan de dialyse verpleegkundigen om op een correcte manier bloedstalen af te nemen bij dialysepatiënten en aan de techniekers op dialyse om te zorgen voor dialysaat. Voormalig diensthoofd *Prof. dr. em. Raymond Vanholder* wil ik bedanken om destijds mijn doctoraat te mogen starten en om input te geven in mijn onderzoek, net als ons huidig diensthoofd *Prof. dr. Wim Van Biesen*.

Graag wil ik *Prof. dr. Björn Meijers* en *Jetty de Loor* bedanken voor de goede samenwerking Gent-Leuven en om mij te laten kennis maken met jullie gloednieuw UPLC tandem MS toestel en nieuwe techniek voor de *high throughput* staalvoorbereiding van uremische toxines.

Prof. dr. Alain Verstraete wil ik bedanken om me te laten verdiepen in de ontwikkeling en validatie van een chromatografische methode. Ik heb hier veel geleerd en hoop dit te kunnen gebruiken in mijn verdere carrière. Ook bedankt om regelmatig samen naar een oplossing te zoeken en bij het toestel te brainstormen, ondanks je drukke agenda en vele buitenlandse missies.

I would like to thank *Prof. dr. Daniel 'Danny' Schneditz* for the pleasant stay in the beautiful city of Graz, Austria. Thanks for your kind invitation and the good collaboration. It was very

interesting to study the distribution of protein-bound uremic toxins in erythrocytes together with you.

Alle donoren die vrijwillig bloed afgestaan hebben voor mijn experimenten wil ik ook even in de kijker plaatsen. Hierbij dank ik alle collega's en vrienden om zich regelmatig te laten prikken, alsook de dialysepatiënten om deel te nemen aan de verschillende studies.

Verder wil ik een woord van dank richten aan al mijn vrienden om me de afgelopen vier jaar bij te staan en te zorgen voor de nodige ontspanning op momenten dat een experiment niet uitdraaide zoals gepland. Bedankt voor jullie begrip in de laatste maanden van mijn doctoraat waarbij ik minder tijd kon maken om mekaar te zien. We halen dit zeker nog in! ;-)

Mijn ouders wil ik graag bedanken om tien jaar geleden mij de mogelijkheid te geven om te starten aan de UGent. Hoewel het eerste jaar een moeilijke start kende, ben ik er toch in geslaagd om vandaag met enige trots mijn doctoraat te kunnen afronden. Moest ik destijds niet gestart zijn en niet volgehouden hebben, ging dit nooit verwezenlijkt kunnen worden. Ook mijn zus *Stefanie* en *schoonfamilie* wil ik danken om af en toe te polsen hoe het met mijn onderzoek ging. Daarnaast ben ik ook mijn petekind *Lars* dankbaar voor de leuke momenten en wil ik zeggen dat ik vanaf nu terug wat meer tijd kan maken.

Tenslotte wil ik mijn wederhelft, *Debbie*, bedanken voor haar luisterend oor, helpende hand en groot hart. Gelukkig heb jij eerder ook een doctoraat afgerond en had je enorm veel begrip voor mijn weinige tijd in de laatste maanden van mijn doctoraat. Het was geen gemakkelijke taak om het huishouden en de zorg van onze prachtige dochter *Lotte* volledig op jou te nemen de afgelopen maanden. *Lotte*, jij bent een schat van een kind en zorgde steeds voor een glimlach op mijn gezicht na een lange werkdag. Het schrijven van deze thesis was weliswaar niet evident met jou in de buurt en daarom hebben we wat minder tijd samen kunnen doorbrengen. Gelukkig kunnen we nu als gezin deze verloren tijd inhalen en samen uitkijken naar jouw broertje of zusje.

Olivier Deltombe

Juni 2018, Gent.

Curriculum vitae

Personal

Name: Olivier Deltombe

Address: Bergbosstraat 25, 9820 Merelbeke

Mobile phone: +32 472 96 61 27

E-mail: olivier.deltombe@hotmail.com

Date of birth (place): July 11, 1990 (Torhout)

Nationality: Belgian

Education

May 2018 – present: **Junior Associate** at pi life sciences consultancy, Ghent, Belgium
Project engineer analytical method validation for equipment cleaning at Pfizer Puurs

Apr 2014 – April 2018: **PhD student in medical sciences**, Ghent University, Belgium
Kinetics of protein-bound uremic toxins in chronic kidney disease
Promoters: Prof. dr. Sunny Eloot and Prof. dr. Annemieke Dhondt

Aug 2013 – Dec 2013: **IWT grant application**, Ghent University, Belgium
Inductively coupled plasma – mass spectrometry (ICP-MS) as detection method in quantitative metabolite profiling
Promoters: Prof. dr. Frank Vanhaecke and Prof. dr. Frédéric Lynen

2008 – July 2013: **Master of Science, Chemistry**, Ghent University, Belgium (cum laude)
Master thesis: *The influence of menstrual blood loss on the isotopic composition of Fe, Cu and Zn in whole blood via MC-ICP-MS*
Promoters: Prof. dr. Frank Vanhaecke and dr. Karen Van Hoecke

2002 – 2008: **Mathematics – sciences**, Sint Jozefhumaniora, Bruges, Belgium

Ghent University Doctoral Schools courses 2014 – 2018

Specialist courses: Pharmacokinetics
Internal training nephrology
Clinical studies: study design, implementation and reporting

Transferrable skills: Authentic networking
Job market for young researchers 2017
Knowledge 2 connect lunch meetings
Advanced academic English: writing skills (life sciences and medicine)
Statistical analysis with SPSS

A1 publications

- Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S (2015) Exploring Protein Binding of Uremic Toxins in Patients with Different Stages of Chronic Kidney Disease and during Hemodialysis. Toxins (Basel) 7:3933–3946. doi: 10.3390/toxins7103933

- Deltombe O, de Loor H, Glorieux G, Dhondt A, Van Biesen W, Meijers B, Eloot S (2017) Exploring binding characteristics and the related competition of different protein-bound uremic toxins. Biochimie 139:20–26. doi: 10.1016/j.biochi.2017.05.010

- Deltombe O, Dhondt A, Van Biesen W, Glorieux G, Eloot S (2017) Effect of sample temperature, pH, and matrix on the percentage protein binding of protein-bound uraemic toxins. Anal Methods 9:1935–1940. doi: 10.1039/C7AY00054E

- Deltombe O, Mertens T, Eloot S, Verstraete A G Development and validation of an UHPLC – high resolution MS method for the quantification of total and free teicoplanin in human plasma. *Will shortly be submitted*

- Deltombe O, Stove V, Glorieux G, Eloot S Protein binding of vancomycin and teicoplanin and the related competition with protein-bound uremic toxins in plasma from healthy subjects and patients on hemodialysis: an *in vitro* study. *In preparation*

- Deltombe O, Glorieux G, Masereeuw R, Schneditz D, Eloot S Selective transport of protein-bound uremic toxins in erythrocytes. *Will shortly be submitted*

Oral presentations

- Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S: Exploring protein binding of uraemic toxins in patients with different stages of chronic kidney disease and during haemodialysis. Oral presentation at the EUTox research meeting (Marseille, France, March 27, 2015)

- Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S: Exploring protein binding of uraemic toxins in chronic kidney disease and haemodialysis patients. Oral presentation at the 42nd European Society for Artificial Organs conference (Leuven, Belgium, September 4, 2015)

- Deltombe O, Dhondt A, Glorieux G, Eloot S: Exploring protein binding of uremic toxins in healthy and uremic blood. Oral presentation at the EUTox research meeting (Ghent, Belgium, March 24, 2017)

- Deltombe O, de Loor H, Glorieux G, Dhondt A, Van Biesen W, Meijers B, Eloot S: Exploring protein binding of different uremic toxins. Oral presentation at the 44th European Society for Artificial Organs conference (Vienna, Austria, September 9, 2017)

- Deltombe O, Dhondt A, Glorieux G, Eloot S: Kinetics of protein-bound uremic toxins in chronic kidney disease: a PhD summary. Oral presentation at the EUTox research meeting (Utrecht, The Netherlands, March 16, 2018)

Poster presentations

- Deltombe O, Van Biesen W, Glorieux G, Massy Z, Dhondt A, Eloot S: Exploring mechanisms of protein binding of uraemic toxins in CKD 2-5 and haemodialysis patients. Poster presentation at the 52nd European Renal Association – European Dialysis and Transplantation Association Conference (London, The United Kingdom, May 29, 2015)

- Deltombe O, de Loor H, Glorieux G, Dhondt A, Van Biesen W, Meijers B, Eloot S: Exploring binding characteristics and related competition of different uraemic toxins. Poster presentation at the Research day and Student research symposium of the Faculties of medicine and health sciences and pharmaceutical sciences (Ghent, Belgium, April 20, 2017)

- Deltombe O, de Loor H, Glorieux G, Dhondt A, Van Biesen W, Meijers B, Eloot S: Exploring binding characteristics and related competition of different protein-bound uraemic toxins. Poster presentation at the 54th European Renal Association – European Dialysis and Transplantation Association Conference (Madrid, Spain, June 5, 2017)